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DISSERTAÇÃO DE MESTRADO

TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Bioremediation for mitigation and recovery from oil spill incidents

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M.ICBAS **2017**

SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE CIÊNCIAS

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BIOREMEDIATION FOR MITIGATION AND RECOVERY FROM OIL SPILL INCIDENTS

Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Acknowledgments

First, I would like to thank my supervisor, Ana Paula Mucha, for all the time and dedication given throughout this past year, following every step of the way and being accessible to discuss all my questions and doubts. I also thank for this opportunity to work in petroleum bioremediation at EcoBioTec laboratory.

I would also like to thanks Marisa Almeida for all the help and guidance throughout this work.

To Fátima Carvalho for the availability to help me and elucidate me in some questions of this work. With the guidance of these 3 researchers, this past year's work allowed me to obtain more knowledge in the area of bioremediation and grow professionally.

I would like to thank my colleagues from EcoBioTec lab, specially to Diogo, Filipa, Inês and Joana, for their help and support given this past year, for always being present to answer some of my doubts, but also for all the sympathy, patience, laughs, craziness and good vibes, throughout this year. I couldn't have asked for better lab partners, myself.

To my best friend, Mafalda Coutinho, who has been by my side for several years, in the good and bad moments, for her friendship, support, laughter and crazy moments shared throughout these years.

To my parents and sisters, for all the love, support and encouragement in my decisions, who have shaped me to become the person I am today. A special thanks to my father, who passed me his love for nature, during my childhood, which helped to form my love for nature, inspired me to follow biology and aspire to work in environmental protection.

This research was partially supported by the research project SpilLess-First line response to oil spills based on native microorganisms cooperation (EASME/EMFF/2016/1.2.1.4/010) supported by the Executive Agency for Small and Medium-sized Enterprises (EASME) delegated by the European Commission, by the structured Program of R&D&I INNOVMAR - Innovation and Sustainability in the Management and Exploitation of Marine Resources (NORTE-01-0145-FEDER-000035), within the R&D Institution CIIMAR (Interdisciplinary Centre of Marine and Environmental Research), supported by the Northern Regional Operational Programme (NORTE2020), through ERDF, and by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020.

Co-funded by the European Maritime and Fisheries Fund

FCT Fundação para a Ciência e a Tecnologia

NORTE2020

PORTUGAL 2020

UNIÃO EUROPEIA Fundo Europeu de Desenvolvimento Regional

Resumo

Derrames de petróleo provenientes de fontes antropogénicas, representam uma grave ameaça para ambientes marinhos, requerendo, por isso, medidas próprias de mitigação. Há, portanto, uma necessidade de se desenvolver técnicas de remediação mais ecológicas e eficientes para o combate a derrames de petróleo. A biorremediação tem sido considerada uma técnica ecológica e promissora, nomeadamente recorrendo ao uso de microrganismos autóctones.

Tendo isto em conta, o presente trabalho tem como objetivos desenvolver consórcios de estirpes bacterianas autóctones degradadoras de petróleo, otimizar a sua fase de enriquecimento com adição de 4 fontes distintas de carbono e testar a eficiência dos consórcios na degradação de hidrocarbonetos de petróleo em estudos de microcosmos, sob tratamentos de biorremediação diferentes com a possibilidade de serem usados para a remediação de derrames de petróleo.

Para tal, 2 consórcios diferentes foram preparados, cada um constituído por uma mistura de 5 estirpes bacterianas, previamente isoladas de um sedimento intertidal de uma praia (Cabo do Mundo) da costa Noroeste Portuguesa, cultivados em meio Bushnell – Haas (BH) e expostas a petróleo. Estes consórcios foram enriquecidos na presença de diferentes fontes de carbono (petróleo e acetato de sódio). Estas fontes de carbono foram selecionadas em testes de enriquecimento com acetato de sódio, petróleo, uma mistura de hidrocarbonetos aromáticos policíclicos (PAHs) e uma mistura de acetato de sódio com PAHs.

Depois, realizam-se experiências de microcosmos em frascos contendo meio BH e petróleo durante 15 dias, sob 4 tratamentos diferentes: atenuação natural, bioestimulação (com adição dos nutrientes N e P), e uma combinação de bioestimulação com bioaumento autóctone usando consórcios enriquecidos tanto com acetato de sódio como com petróleo.

No início e fim da experiência, foram retiradas amostras para estimar a abundância de microrganismos degradadores de petróleo através de um método adaptado do número mais provável e para avaliar a degradação de hidrocarbonetos através da análise das concentrações de hidrocarbonetos totais (TPHs) (por espectrometria de FT/IR). Foi também realizado o isolamento e identificação de estirpes bacterianas degradadoras de hidrocarbonetos, no final da experiência.

O tratamento com o consórcio enriquecido com acetato de sódio provou ser o melhor na degradação de hidrocarbonetos, com abundância elevada de degradadores de hidrocarbonetos e elevada percentagem de remoção de TPHs, tendo sido detetada a

presença no meio das estirpes bacterianas introduzidas do consórcio, no final da experiência.

Este estudo dá uma noção da capacidade natural das comunidades para degradar hidrocarbonetos e da sua aplicabilidade em técnicas de biorremediação que podem ser adotadas em futuros planos de contingência nacionais. Desta forma, será possível atuar local e ecologicamente em resposta a um eventual incidente de derrame de petróleo.

Palavras-chave: Biorremediação, derrames de petróleo, bioaumento autóctone, consórcios bacterianos, ecossistemas marinhos.

Abstract

Oil spills from anthropogenic sources pose a serious threat to marine ecosystems, requiring prompt mitigation measures. There is thus a need for the development of cleaner and more efficient remediation techniques. Bioremediation has proven to be an eco-friendly and promising remediation technique, especially when using autochthonous microorganisms.

In this line, we aimed to develop consortia of autochthonous hydrocarbon-degrading bacterial strains, optimize their enrichment process with 4 different carbon sources, and test the efficiency of the consortia to degrade petroleum hydrocarbons in a microcosm experiment under different bioremediation treatments, which could be used for the bioremediation of oil spills.

Thus, two different consortia were prepared, each constituted by a mixture of 5 bacterial strains previously isolated from a sandy beach (Cabo do Mundo) intertidal sediment in the NW Portuguese coast, cultivated in Bushnell – Haas broth (BH) and exposed to petroleum. These consortia were enriched in the presence of two different sources of carbon (petroleum and sodium acetate). These carbon source were selected from previous enrichment tests with sodium acetate, petroleum, a mixture of the polycyclic aromatic hydrocarbons (PAHs) and a mixture of sodium acetate and the PAHs.

Then, microcosm experiments were performed in flasks containing BH medium and petroleum, for 15 days, under four different treatments: natural attenuation, biostimulation (with addition of N and P nutrients), and combination of biostimulation and autochthonous bioaugmentation with consortia either enriched with sodium acetate or with petroleum. Samples were taken at the beginning and end of the experiment to assess hydrocarbon degrading microorganisms through an adapted most probable number method and for analysis of total hydrocarbons (TPHs) concentrations (by FT/IR spectrometry), to evaluate hydrocarbons degradation at the end of the experiment. Isolation and identification of hydrocarbon-degrading bacterial stains was also carried out at the end of the experiment.

The treatment with the consortium enriched with sodium acetate performed better for hydrocarbons degradation, with high hydrocarbon-degraders abundance, high TPHs removal percentage and the presence of the introduced bacterial strains consortium in the medium at the end of the experiments.

This study provides an insight into the natural community capacity to degrade hydrocarbons and its potential application in bioremediation techniques that can be adopted in the future in national contingency plans. In this way, it is possible to act locally and ecologically facing an eventual oil spill incident.

Keywords: Bioremediation, oil spills, autochthonous bioaugmentation, bacterial consortia, marine ecosystems.

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Abbreviations

A + PAHs	Combination of sodium acetate and polycyclic aromatic hydrocarbons
A	Sodium acetate
ABA	Autochthonous bioaugmentation
BA	Bioaugmentation
BH	Bushnell–Haas
BS	Biostimulation
BS + ABA (EA)	Combination of biostimulation and bioaugmentation with a consortium enriched with sodium acetate
BS + ABA (EP)	Combination of biostimulation and bioaugmentation with a consortium enriched with petroleum
C/N/P	Ratio of carbon, nitrogen and phosphorous
CFUs	Colony-forming units
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
EA	Enrichment with sodium acetate
EP	Enrichment with petroleum
FT/IR	Fourier transformed infrared spectroscopy
HC	Hydrocarbon
Int	Iodonitrotetrazolium Violet
KH_2PO_4	Potassium di-hydrogen phosphate
KNO_3	Potassium nitrate
MPN	Most probable number
N	Natural attenuation
NaCL	Sodium chloride
OD	Optical density
OHCB	Obligate hydrocarbonoclastic bacteria

P	Petroleum
PAHs	Polycyclic aromatic hydrocarbons
PCA	Plate count agar
rRNA	Ribosomal ribonucleic acid
SW	Seawater
TAE	Tris-acetate-ethylenediamine tetraacetic acid
TCA	Tricarboxylic acid cycle
TPHs	Total petroleum hydrocarbons

Chapter 1
General introduction

1. General introduction

1.1. Oil pollution in marine environments

In present days, society is still dependent on fossil fuels for energy production, transports and industry, which demands for a continuous exploitation and transport of petroleum and its derivatives on the sea.

Marine environments are very sensitive to contamination and are constantly targets of petroleum hydrocarbons (HC) pollution, whether is from large-scale oil spills, or smaller ones (less than 7 tons). Oil spills represent an acute form of pollution and can be originated from storage, exploitation in offshore platforms and transportation of petroleum and its products (Brooijmans *et al.*, 2009; Das & Chandran, 2011)

Maritime shipping and transport of petroleum in oil tankers is made across the oceans within a network of routes, which intensifies its frequency of passage and quantities of crude oil transported in near-shore areas (figure 1). Therefore, coastal habitats are at risk and frequently affected by oil spill incidents. The European Atlantic area represents a hotspot for oil tankers routes (Acosta-González *et al.*, 2015; Vieites *et al.*, 2004). Portugal is a susceptible country to oil spills, giving its geographical position and closeness to the previous hotspot trade routes (figure 1).

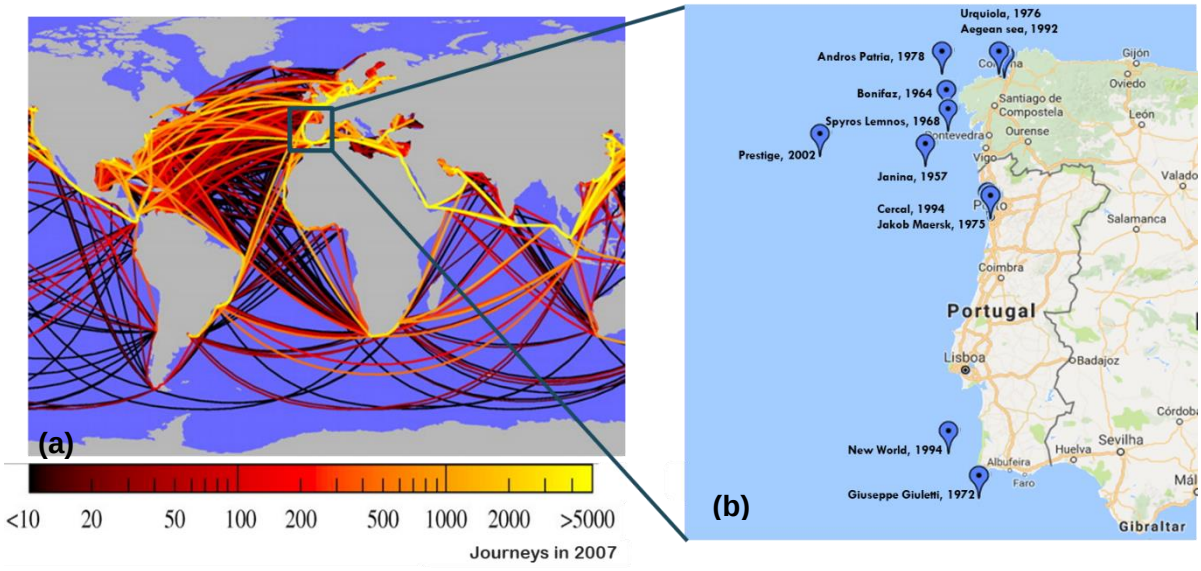

Figure 1 - (a) Routes of all cargo ships bigger than 10 000 GT during the year of 2007, where the color scale indicates the number of journeys along each route (adapted from (Kaluza *et al*., 2010); (b) Oil spill incidents that have occurred in the Galicia and Portugal shore (adapted from http://wwz.cedre.fr/en/Our-resources/Spills/Spill-map).

Although oil spills have a huge negative impact on the environment, they only represent less than 10% of the total petroleum HC input in the environment (Ivshina *et al.*, 2015). The major source of petroleum HC pollution at sea is not derived from oil spill accidents, but from chronic pollution (along time) originated from natural seeps, leaking ships and illegal cleaning of the ship's bilges (Henkel *et al.*, 2014).

These type of contamination is more difficult to control and monitor, leading to a continuous increase of HC pollution in the marine environments (Henkel *et al.*, 2014).

Nevertheless, the amount and concentration of the toxic chemicals after an oil spill are present in such high rates that must be taken seriously.

Oil spills are known to cause severe toxicity and health problems to the living organisms and in many cases, can cause death of marine fauna and flora. Besides, at the sea surface, an oil slick could form, blocking UV radiation as well as the aeration of the ocean, affecting all the life below the polluted water column (Xue *et al.*, 2015). All the prior effects lead to changes in the microbial communities function and structure (Acosta-González *et al.,* 2015) along with negative outcomes on trophic chains. Economy is also affected, as it interferes with fisheries, tourism and recreational activities.

Along the decades we have seen some oil spill disasters and witness its devastating consequences and repercussions until today. The Deepwater Horizon (2010), the Prestige (2002) and the Exxon Valdez (1989) oil spills are some examples that have marked History for their high negative impact on the environment and marine wildlife. In table 1 are represented the 10 biggest oil spills in History.

The spillage of the "Prestige" oil tanker, in November 2002, about 30 miles away from the Galician coast, reached the shore and affected more than 600 beaches. The damages of the spill also reached some parts of Northern Portugal coast (Acosta-González *et al.*, 2015; Medina-Bellver *et al.*, 2005; Morales-Caselles *et al.*, 2008).

Table 1 – Top 10 of the biggest oil spills in history, including year, location and amount of oil spilled (in tons). Information collected from the ITOPF Oil Tanker Spill Statistics 2016 [http://www.itopf.com/knowledge-resources/data-statistics/statistics/] and CEDRE oil spills database [online_ http://wwz.cedre.fr/en/Our-resources/Spills].

TOP 10	NAME OF THE OIL SPILL	YEAR	ACCIDENT AREA	TON
1	Gulf War	1991	Persian Gulf, Iran	1 091 405
2	Deep Water Horizon	2010	Gulf of Mexico	686 000
3	Ixtoc 1	1979	Bay of Campêche, Mexico	471 430
4	Atlantic Express	1979	Off the coast of Tobago, Caribbean	287 000
5	ABT Summer	1991	Off the coast of Angola	260 000
6	Nowruz	1983	Persian Gulf, Iran	260 000
7	Castillo Bellver	1983	Off the coast of Table Bay, South Africa	252 000
8	Amoco Cadiz	1978	Portsall, France	223 000
9	Haven	1991	Off the coast of Genoa, Italy	144 000
10	Odyssey	1988	Off Nova Scotia, Canada	132 000

Once the oil reaches the sea, it can have different behaviors and fates depending on the climatic conditions, hydrodynamics and location where the spillage occurred (Ivshina *et al.*, 2015; Shetaia *et al.*, 2016) in addition to its mass, composition and chemical properties.

The bioavailability of HC for degradation in the sea changes as the oil is at its dispersed, floating or settled form (Boglaienko & Tansel, 2016).

The oil undergoes several weathering processes like evaporation, dissolution, dispersion, sedimentation, photo-oxidation and biodegradation (Acosta-González *et al.*, 2015; Gong *et al.*, 2014; Mishra & Kumar, 2015). These processes will have an impact on the success of remediation techniques facing an oil spill.

The composition and type of the oil will also have an influence in the process of degradation. Crude oil is a complex mixture of petroleum HC including 4 groups of chemicals: alkanes, aromatic HC, resins and asphaltenes (Das & Chandran, 2011), represented in the figure 2.

Alkanes are linear and cyclic hydrocarbons only constituted by single carbon bonds. Aromatic HC are constituted by one or more benzene rings, being divided into monocyclic and polycyclic aromatic hydrocarbons (PAHs). Resins and asphaltenes, on the other hand, are polar compounds, with complex structures composed by carbon and hydrogen, with the addition of elements like nitrogen, sulfur and oxygen (Varjani & Upasani, 2016).

Crude oil can be classified into light, medium and heavy oil, accordingly to the proportion of the different components. A light oil has high content of alkanes and aromatic HC and lower concentrations of resins and asphaltenes. In opposite, heavy oil has higher proportion of resins and asphaltenes (Hassanshahian & Cappello, 2013).

Some oil components, like high-molecular weight PAHs and resins, are recalcitrant and extremely toxic to marine organisms, and could persist in the environment for decades (Hassanshahian & Cappello, 2013; Leahy & Colwell, 1990).

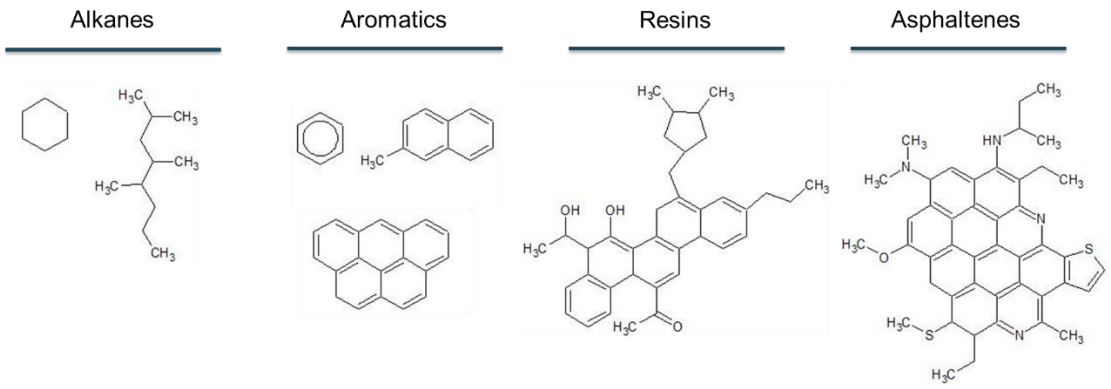

Figure 2 - A representation of some of the molecular structures of alkane hydrocarbons, aromatic hydrocarbons, resins and asphaltenes. Structures were drawn in the program ACD/ChemSketch (Freeware) 2016.2.2.

Despite of the weathering processes and physic-chemical changes of the oil, an important part of oil degradation in the environment is carried by microorganisms capable of degrading petroleum HC.

1.2. Degradation of hydrocarbons by marine microorganisms

Some marine microorganisms such as fungi, yeasts and bacteria are capable of degrading petroleum HC. In marine environments bacteria is thought to be the key oil-degrading organisms within microbial communities (Leahy & Colwell, 1990).

When in an oil-contaminated environment, the microbial community suffers an adaptation process, occurring the activation of enzymes, selective pressure, alterations in the genes by gene transfers and mutations linked to the oil-degrading metabolism (Leahy & Colwell, 1990).

These organisms use the carbon from the oil as a source of energy, breaking its compounds into others with lower molecular weight (Guo-liang *et al.*, 2005). The HC degradation usually

starts from low-molecular weight HCs (linear alkanes) to higher ones (cyclic alkanes or aromatic HC) and from simple structures to more complex ones (Leahy & Colwell, 1990; Li *et al.*, 2016; Varjani & Upasani, 2017).

The number and weight of the atoms determines the molecular weight of a compound. Considering the HC structure, linear, branched, cyclic or aromatic, the susceptibility of the HC for biodegradation will change (Das & Chandran, 2010). Like n- alkanes, aromatic HC with low molecular weight, such as toluene and benzene, can be easily degraded by microorganisms. For n-alkanes, a wide bacterial species can degrade it, but, the more complex a HC molecular structure is, in general, the more difficult is for the microorganisms to degrade, and so, fewer species can degrade them (Atlas, 1995; Varjani, 2017). It is difficult to find species that can degrade all HC.

So, given the complexity of the crude oil, and since different bacterial strains can degrade a specific group of HCs, a diverse microbial community will be able to degrade a much larger set of HCs, thus delivering a better performance in the crude oil degradation (Dell'Anno *et al.*, 2012; Hassanshahian & Cappello, 2013).

In the degradation process, the bioavailability of HC is very important, since many times bacteria cannot have access to the hydrophobic part of the oil. So, to work around that some bacterial strains can produce and secrete extracellular enzymes that act as natural surfactants (biosurfactants), such as glycolipids, phospholipids, lipopeptides and lipoproteins (Fuentes *et al.*, 2014; Kleindienst *et al.*, 2015; Marti *et al.*, 2014).

These biosurfactants reduce the water-oil tension, disperses the oil into small droplets and increases the oil surface area, increasing, therefore, HC availability to the organisms (Das & Chandran, 2011; Paul *et al.*, 2005). Another method that some bacterial strains adopt is the adhesion to the oil-water interface, that allows an easier uptake of the HCs by the cells (Abbasnezhad *et al.*, 2011).

For the degradation of the compounds, bacteria require a certain amount of nutrients, specially nitrogen and phosphorous, that lacks in the marine environment in the proportion needed after an oil spill (Hassanshahian & Cappello, 2013; Ron & Rosenberg, 2014). The Redfield ratio of C/N/P in the oceans defines values of 106:16:1, meanwhile in bioremediation studies a ratio of 100:10:1 (C/N/P) is used for the optimization of nutrients in the assays (Almeida *et al.*, 2013; Nikolopoulou*, et al.*, 2013).

Once the cells uptake HC, they could be metabolized either in aerobiose or anaerobiose pathway (figure 3). An ideal degradation process of the HC degradation by microorganisms ends with the release of CO_2 (mineralization of HC), biomass and other non-toxic water-soluble products (Hassanshahian & Capello, 2013).

In aerobiose pathways, the first step is the activation of the HC substrate by oxigenases, with the conversion to alkanols, in the case of alkanes, and catechols, in case of aromatic HC degradation. Further metabolism with β-oxidation and intermediates entering the tricarboxylic acid cycle produces energy, biomass and releases CO_2 (Atlas, 1995; Das & Chandran, 2011; Hassanshahian & Cappello, 2013; Varjani, 2017; Varjani & Upasani, 2017). As for the anaerobic pathway, not much is yet known, but a first activation phase with addition of fumarate, carboxylation for both aliphatic and aromatics occurs, with further metabolism, like decarboxylation, ring cleavage and β-oxidation (Foght, 2008; Hassanshahian & Cappello, 2013; Sierra-Garcia & de Oliveira, 2013).

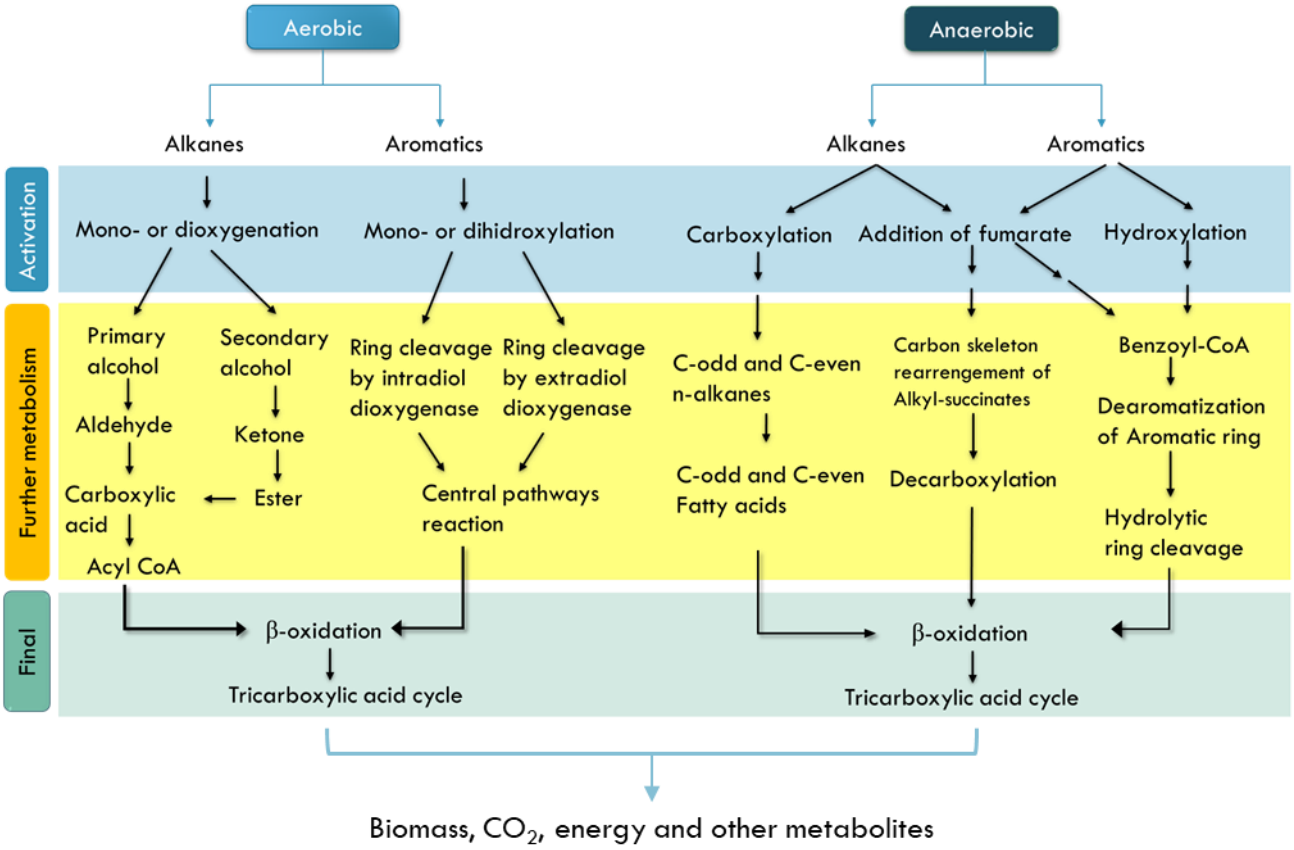

Figure 3 - Principles of the degradation of hydrocarbons by aerobic and anaerobic pathways in a scheme, adapted from Sierra-Garcia & de Oliveira, 2013.

Aerobic conditions are considered the most efficient form of HC degradation, since it has a faster degradation pathway (figure 4) and facilitates the action of oxygenases in the compounds, strong oil-degrading enzymes (Fuentes *et al.*, 2014; Venosa & Zhu, 2003).

Degradation of petroleum HC in anaerobiose, has been considered not significant in the process, but recently is thought to be an important part in the degradation of some aromatic HC, under certain conditions (such as sediments and in lower zones of the water column) (Leahy & Colwell, 1990; Venosa & Zhu, 2003). This process can happen in sulfate-reducing conditions and methanogenic conditions (Berdugo-Clavijo & Gieg, 2014; Foght, 2008).

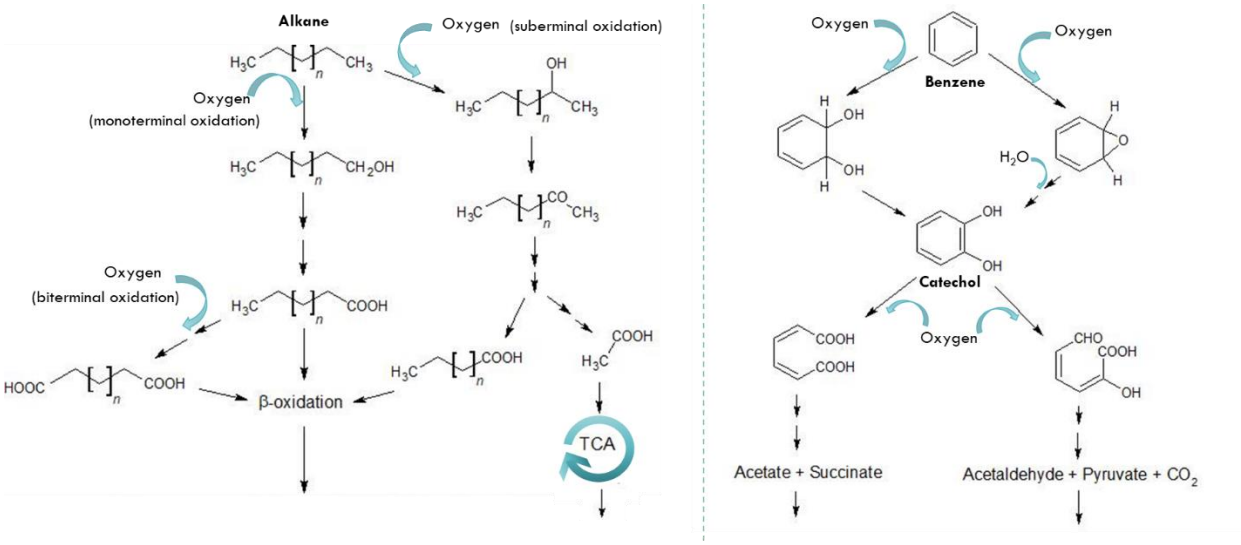

Figure 4 - Aerobic degradation pathways of aliphatic and aromatic hydrocarbons, alkane and benzene molecules, adapted from Hassanshahian & Capello, 2013. TCA – tricarboxylic acid cycle.

Yakimov *et al.* (2007) distinguishes the crucial part of "obligate hydrocarbonoclastic bacteria" (OHCB), known for their exclusive feeding on hydrocarbons, in the cleaning process of HC polluted sites.

Alcanivorax borkumensis is an example of such OHCBs, with a genome rich in oil-degrading enzyme genes and pili that allows the attachment to the water-oil surface area and facilitates the HC degradation (Brooijmans *et al.*, 2009). Kasai *et al.* (2002) notice the predominance of the genus *Alcanivorax* within the microbial community in studies with water contaminated with crude oil in the presence of phosphorous and nitrogen. Within the OHCB, we can find organisms from the genera *Oleispira*, *Thalassolituus* and *Cycloclasticus* (Yakimov *et al*., 2007).

Associated with PAHs degradation there are organisms from the genera *Arthrobacter*, *Sphingomonas*, *Pseudomonas*, and *Rhodococcus* (Brooijmans *et al.*, 2009).

Rhodococcus erythropolis has been continuously linked with the degradation of petroleum HC in several studies (de Carvalho & da Fonseca, 2005; Martinkova *et al.*, 2009). The genera *Rhodococcus* is known to have mycolic acids in the composition of its external cell layer, which allows the adhesion of the cell to the water-oil interface and a variety of oil degrading enzymes with wide biodegradation capacities (Fuentes *et al.*, 2014; Larkin *et al.*, 2005; Martinkova *et al.*, 2009).

Rhodococcus erythropolis, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Micrococcus luteus* are some of the biosurfactants producing species, their surfactants having the potential to be applied in oil biodegradation processes (Makkar *et al.*, 2011). Regardless of their natural capacity to degrade HC, in case of an oil spill, natural microbial communities cannot act nor degrade fast enough to prevent the negative consequences in the environment towards organisms, either from the lack of needed nutrients or low bioavailability of the oil constituents.

Thus, there is a need to implement remediation techniques to face an oil spill.

1.3. Remediation technologies (clean-up)

Facing an oil spill disaster, quite a few remediation techniques can be performed, including mechanical, chemical and biological ones. The type, amount and location of the oil spilled will command the clean – up efforts in the remediation processes.

Current oil spill clean-up steps focus on mechanical removal and on the use of chemical dispersants, which can cause additional contamination to the environment and marine organisms (Al-Majed *et al.*, 2012; Ivshina *et al.*, 2015).

1.3.1. Mechanical recovery

The usual first-line response to an oil spill occurring in open sea includes the skimming of the oils top layer with the help of boats and boomers. With this technique, it is intended to remove as much oil as possible from the sea.

But this technology cannot remove all the oil from the sea surface and is not suitable to be applied neither in case of a large oil spill nor in open sea with difficult access of boats and humans (Nikolopoulou & Kalogerakis, 2008; Prince *et al.*, 2003).

Combining with this technique, in-situ burn of oil is sometimes applied. This is a very polluting method, leading to the emission of massive amounts of volatile compounds, such as CO_2 and PAHs, and the consequent contamination of the environment. Beyond the disadvantages of in-situ burn, it only removes the lower-weight HC, leaving the higher ones in the environment (Ivshina *et al.*, 2015).

1.3.2. Chemical dispersants

Another recurrently used method is the application of chemical dispersants to the oil surface. Chemical dispersants are a combination of solvents and surfactants that allows the dispersion of the oil into droplets, making HC compounds more available to be degraded by the natural microbial community (Kirby & Law, 2008; Kleindienst *et al.*, 2015; Nikolopoulou & Kalogerakis, 2008).

Surfactants accelerates the biodegradation rate, but when used in high concentrations it can lead to growth inhibition and phenotypic changes in HC degrading microbial communities as demonstrated by Tian *et al.* (2016).

The assortment of a surfactant depends on the type of habitat to be remediated and its composition and efficiency. This technique is considered ideal to be applied for some environments such as deep water and hostile weather conditions (Ivshina *et al*., 2015).

Even though this technique is widely used, it can sometimes be toxic to some marine organisms and lead to the aggregation of the oil-droplets and sedimentation (Barron, 2012; Bravo-Linares *et al.*, 2011; Hemmer *et al.*, 2011; Kirby & Law, 2008; Seidel *et al.*, 2016).

In alternative, surfactants can be originated from natural sources, being produced by some bacterial strains when in the presence of the oil, as described in the previous section. The application of biosurfactants as a green-remediation technology is currently being discussed (Marti *et al.*, 2014).

Comparing to the chemical surfactants, biosurfactants are more biodegradable, have higher selectivity and can act in environments with extreme conditions of temperature, salinity and pH (Souza *et al.*, 2014).

1.3.3. Bioremediation as a promising remediation technology

The current response mechanisms may not remove completely the oil spilled and remediate the environment. Only mechanical and chemical methods are not sufficient to treat affected environments and assure the ecologic equilibrium of the environment. Bioremediation might be a suitable method to fill up the gaps in current remediation technology.

Bioremediation has been increasingly considered a cost-effective, efficient and ecologic tool for remediating oil polluted sites, since it does not cause negative environmental impacts,

contrarily to the commonly used methods, and can lead to a faster HC compounds degradation (Almeida *et al.*, 2013; Li *et al.*, 2016; Prince *et al.*, 2003; Priya *et al.*, 2016).

Bioremediation is based on the natural ability of some microorganisms, such as bacteria, to degrade HC and is commonly divided into two strategies: biostimulation (BS) and bioaugmentation (BA).

Biostimulation is the process in which the indigenous microbial community is enriched by the addition of essential nutrients, like nitrogen and phosphorous, leading to an increase in the oil degradation. On the other hand, bioaugmentation promotes the enhancement of the HC degradation, through the addition of bacterial strains, isolated or in a consortium, capable of degrading HC (Lee & Mora, 1999; Pontes *et al.*, 2013).

The efficiency of the bioremediation processes is limited by several factors such as temperature, nutrients, oxygen, composition and concentration of petroleum HCs and microbial community structure. During degradation, natural microbial communities often lack essential nutrients, like nitrogen and phosphorous, needed for bacteria metabolism. Aiming to fill this gap, water-soluble fertilizers can be used as a source of these nutrients (Nikolopoulou & Kalogerakis, 2008).

Other fertilizer can be used. For instance, Ron and Rosenberg (2014) propose the use of a natural fertilizer, uric acid (guano), as a fertilizer in oil spills remediation, since it is rich in nitrogen and phosphorous and is easily available in the market.

Besides the lack of nutrients, the low bioremediation rate in an oil spillage might be related to the lack of oil - degraders in the natural microbial community. In this case, bioaugmentation techniques can be implemented.

Relatively to bioaugmentation, exogenous and autochthonous bioaugmentation (ABA) can be considered, which represents the input of exogenous oil-degrading bacteria to the medium, or the input with indigenous oil-degrading bacteria, respectively.

Several scientists investigated the bioaugmentation approach in oil-contaminated environments and through a selection and isolation of the species with this capacity some products (microorganisms) are currently commercialized (Tyagi *et al.*, 2011).

Some marine oil-degrading species, such as *Alcanivorax borkumensis*, *Marinobacter hydrocarbonoclasticus*, *Cycloclasticus pugetii* and *Oleispira antarctica*, are studied and available for their known HC degrading capacity (Mapelli *et al.*, 2017).

The use of autochthonous microorganisms can be advantageous, since they are better adapted to the affected environment, leading to a better efficiency in the oil degradation. This strategy can be more successful than the input of exogenous microorganisms, which

commonly cannot compete with the natural microbial community, not prosper and not enhance the HC degradation (Fodelianakis *et al.*, 2015; Tao *et al.*, 2017). In addition, the use of autochthonous microorganisms avoids the unpredictable ecological impacts that can lead from the introduction of non-native organisms. The importance and success of ABA has been increasing in recent studies and its possible application in the field is being considerate (Hosokawa *et al.*, 2009; Mapelli *et al.*, 2017; Nikolopoulou*, et al.*, 2013).

When applying BA treatments, the use of a consortium of bacterial strains can be more effective in the degradation of the petroleum HC, as each strain degrades different oil compounds, thus leading to a broader spectrum of action (Bacosa *et al.*, 2012; Vila *et al.*, 2010). Vila *et al.* (2010), observed the structural dynamics of an enriched microbial consortium, where species from diverse groups were involved in the degradation of different HC compounds, leading to an efficient oil degradation.

The implementation of bioremediation in a real-case scenario of an oil-spill is still at its beginning, and since there are a lot of variables in the environment, the effect of bioaugmentation is difficult to predict. But, before applying to the oil spill, bioremediation techniques must be previously tested in real-scenario simulated environment conditions, such as mesocosms and field tests (El Fantroussi & Agathos, 2005; Venosa *et al.*, 1996).

Nevertheless, some cases, like the cleanup of the crude oiled beach, after the Exxon-Valdez oil spill, where fertilizers were used to stimulate the degradation of HC by the natural microbial community, can be taken as examples of the success and validation of bioremediation technology (Gertler *et al.*, 2009; Lindstrom *et al.*, 1991).

A combined effect of the techniques of biostimulation and bioaugmentation, could enhance the HC degradation rate, and should be more deeply studied (El Fantroussi & Agathos, 2005; Nikolopoulou *et al.*, 2013), particularly the autochthonous bioaugmentation.

1.4. Current Portuguese national contingency plan

There is some legislation in Portugal to act in case of an oil spill. The clean-up process is headed and performed by the national maritime authority, within the project “Mar Limpo” (http://www.amn.pt/DCPM/Paginas/oquee.aspx).

In case of an oil spill incident, they establish various levels of danger, from the 4º, with a local action, to the 1º degree, with a national action, facing the oil spill and a more dangerous situation.

To deliver an efficient response to an oil spill, they adopt a preparation phase, where they maintain the materials needed for the response, educate and train the personnel involved and perform drills where an oil-spill is simulated. When an oil spill occurs, maritime authorities, specialists, state personnel and even volunteers can be involved in the response actions.

In case of an oil spill, first there must be the confirmation of spill, a registration of the facts along the response procedure and the alert of the responsible entities. This is followed by the containment of the spill and the proper intervention, depending on the affected site.

The maritime authority has at their service, boomers, skimmers, chemical dispersants, pumps, containers absorbents and maritime vehicles and the commonly used clean-up technologies, and software which can simulate oil behavior, calculate its trajectories as well as software that can track oil tankers. They lack, however, some bioremediation technology.

So, there is a need for more studies on the autochthonous biodegradation and deeper knowledge of the oil-degraders along the Portuguese coast and seas, as well as the development of suitable consortia, from microcosms, mesocosms or field tests, to act in case of an oil spill.

The incorporation of the autochthonous bioremediation in the Portuguese national contingency plan, would provide a more efficient and ecologic response to an oil spill.

1.5. Objectives

Marine environments are rich in biodiversity, presenting different microbial communities depending on the habitat. Some microorganisms are known to produce compounds, participate in the geochemical cycles and in the degradation of numerous compounds, for instance, petroleum HC.

The selection of oil-degrading bacteria happens when a microbial community is in contact with petroleum: the strains able to use the HC as their energy source, will prosper in the environment and remain in it, while the strains that cannot survive in that habitat, for lack of nutrients and other energy sources or toxicity of the oil, perish and are not present in the contaminated habitat.

This work had three main objectives, the first one (1) was to develop an autochthonous oil-degrading consortium with previously isolated bacterial strains with HC degradation potential, (2) secondly, optimize an enrichment process of that consortium with 4 different carbon sources, and finally (3) evaluate the efficiency of the consortium to degrade

petroleum HC, in a microcosm experiment, using different bioremediation treatments, the biostimulation and a combination of biostimulation with bioaugmentation.

Towards these aims, 5 bacterial strains, previously isolated, were tested, alone and in a mixture, for their ability to degrade petroleum HC, and then tested in enrichment experiments with 4 different carbon sources. The best performing consortia, in terms of abundance of oil degraders, was then tested in microcosms experiments in which the percentage of removal hydrocarbons and oil degraders abundance was evaluated.

This thesis is organized by a first chapter of introduction, followed by a description of the materials and methods used in the different experiments, a chapter of the results followed by its discussion and the final conclusions with some future perspectives.

Chapter 2
Materials and Methods

2. Materials and Methods

2.1. Selection of an autochthonous hydrocarbons-degrading bacteria consortium

Aiming the selection of an autochthonous hydrocarbons-degrading bacterial consortium, 5 bacterial strains previously isolated from a sandy sediment beach were tested for their ability, alone and in a mixture, to degrade petroleum hydrocarbons (HC), by the most probable number (MPN) method. Then enrichment experiments were carried out with an even mixture of the 5 bacterial strains in which 4 different carbon sources were tested. Different carbon sources were tested, to optimize the conditions for biomass production and its possible application in oil remediation biotechnology. Later, a microcosm study with different treatments, simulating natural attenuation process and remediation techniques was performed.

2.1.1. Sampling site of the bacterial strains and hydrocarbons contamination history

The bacterial strains (CPN 1, 2, 3, 4 and 5) were isolated in a previous work (Gouveia, 2015), from a microbial consortium that showed potential to degrade petroleum HC. This microbial consortium was obtained from a sandy sediment collect in a northwestern Portuguese beach, “Cabo do Mundo” (41°13'13.9"N 8°42'53.1"W), near Matosinhos city. This beach is located near an oil refinery (Petrogal, Matosinhos) and about 4 km from the Leixões Harbor (Figure 5). The refinery represents a risk for HC contamination, as observed in August 1994 and in 2007, when surrounding beaches were polluted due to malfunction of pipelines and escapes of residues from its wastewater treatment plant to the ocean. Moreover, several ships pass near this beach due to the proximity of the harbor. In 1975, the Jakob Maersk oil spill has occurred in the harbor of Leixões, being considered one of the biggest oil spills accidents, in the 13º place, according to the ITOPF statistics, affecting all nearby beaches.

This area was chosen for study, given the risk accounted in this area and its HC pollution history that might have selected a HC degrading microbial community in its sediments.

In the work of Gouveia (2015) samples were collected from the beach Cabo do Mundo, and exposed to crude oil in a medium supplemented with N and P in a 15 days microcosms

study. At the end, samples from each flask were taken and grown in agar plates. Obtained bacterial strains were isolated and stored at -80 ºC.

Figure 5 - Location of beach "Cabo do Mundo" from which CPN samples were isolated, the oil refinery "Petrogal", marked in red in the map and the Leixões harbour (from Google Maps).

2.1.2. Growth of isolated bacterial strains

Bacterial strains were taken from preservation at -80 ºC, unfrozen and 100 μL samples of each were collected and spread in a general-growth medium, Plate count agar (PCA), in sterilized petri plates. The plates were inoculated at 28 ºC, for about 3 days, until growth was achieved. Consecutive streaking method was applied to each plate to acquire pure colonies (following the presupposition that one colony was originated from one initial cell).

Once pure colonies have grown, samples were collected for DNA extraction. For that, one loop of each isolate's colony was collected and re-suspended in 1 mL of sterile saline solution (85 % v/v) in 2 mL microtubes. Afterwards, the tubes were centrifuge for 5 min at 7 G, the supernatant being discard and the pellet store at -20 ºC, for further DNA extraction and species identification.

All material and mediums used were sterilized, by autoclaving at 121 ºC, and manipulations were carried out in a flow-chamber with an initial 20 min UV decontamination cycle to ensure sterile conditions and avoid microbial contaminations.

2.1.3. Preparation of a hydrocarbons-degrading bacterial consortium

Each bacterial strain was obtained by collecting 2 loops of each pure colony (section 2.1.2), which was re-suspended in 1 mL of sterilized Bushnell–Haas (BH) medium, in 2 mL microtube. The BH medium is a mineral-salt, commonly used for HC degradation evaluation, supplemented with NaCl (2 % (v/v)) to simulate a marine solution. The optical density (OD), measured at 600 nm in a spectrophotometer (V-1200 Spectrophotometer, VWR), of each solution was adjusted to ca. 0.1, through sequential dilutions with sterilized BH medium. For the mixture of the 5 isolated bacterial strains (MIX), equal amounts of each strain were transferred into a 2 mL microtube with 1 mL of sterilized BH medium. Similar sequential dilutions were made to reach an initial OD of ca. 0.1.

The abundance of hydrocarbons-degrading bacteria was estimated in each bacterial strain solution and in the MIX by the MPN method. The MIX was then tested for their growth in BH medium enriched with different carbon sources (section 2.1.4).

All material used was sterilized as before and manipulations were carried out in a flow-chamber, with an initial 20 min UV decontamination cycle, to ensure sterile conditions and avoid microbial contaminations.

2.1.4. Enrichment experiments with 4 different carbon sources

The enrichment experiments were performed in 100 mL sterilized glass flasks, with 10 mL of sterile BH medium inoculated with 100 μL of the MIX solution and 4 different carbon sources: petroleum (P); sodium acetate (A); a mixture of the polycyclic aromatic hydrocarbons (PAHs) naphthalene, anthracene, fluoranthene and pyrene; and a mixture of sodium acetate and the 4 PAHs (A+PAHs), with a final concentration of 0.01, 0.1, 0.01 and 0.11 % of carbon relatively to the medium, respectively.

The addition of the MIX solution to the flasks was done in a decontaminated flow-chamber, to ensure sterile conditions and avoid microbial contaminations. The addition of a 100 μL of a sterilized 10 % sodium acetate solution to the flasks A and A+PAHs was also performed in the flow-chamber.

For the solution of PAHs, 0.1 g of each PAH was weight and dissolved in 10 mL of dichloromethane and subsequently 100 μL were transference to the respective flasks. In the P flasks, 100 μL of petroleum, filtered through 0.2 μm sterile cellulose acetate membrane filters (VWR) with a 1 mL syringe, was added to each flask. The addition of petroleum and PAHs to the flasks was performed in an extractor hood.

Each treatment was tested in triplicate. The experiment lasted 15 days, the flasks being with lids closed, under constant agitation and at constant temperature (100 rpm at 28 ºC). At the end of the experiments samples were collected for MPN evaluation.

2.1.5. Abundance of hydrocarbons degraders by the Most Probable Number Method

A MPN method adapted from Wrenn and Venosa (1996) was used to evaluate the abundance of hydrocarbons-degrading bacteria.

This test is performed in 96 well-plates (Figure 6a), with petroleum as the only carbon source added and BH as a culture medium. In the lines A and C to G, 180 μL of sterilized BH medium supplemented with NaCl (2 % v/v) was added, except for the A1 well, where 180 μL of the initial sample was put. Throughout the line A, tenfold dilutions were applied by pipetting 20 μL from the former well. The last well stayed only with BH medium, being used as control.

Next, 10 μL of petroleum, filtered by 0.2 μm sterile cellulose acetate membrane filter (VWR), was added to all the wells in the lines C to G. With a multichannel 200 μL pipette, 20 μL of the line A wells were pipetted to the lines C to G, obtaining for each dilution 5 replicates.

The plates were incubated for 15 days at room temperature. At the end of the incubation period, wells from lines C to G were dyed with 50 μL of a 3 mg L^{-1} Iodonitrotetrazolium Violet (INT) solution, sterilized by filtration through 0.2 μm sterile cellulose acetate membrane filters (VWR). This dye turns purple when in the presence of HC degradation products and a day after the coloration (Figure 6b) positive wells are counted and calculated in the MPN method calculator program.

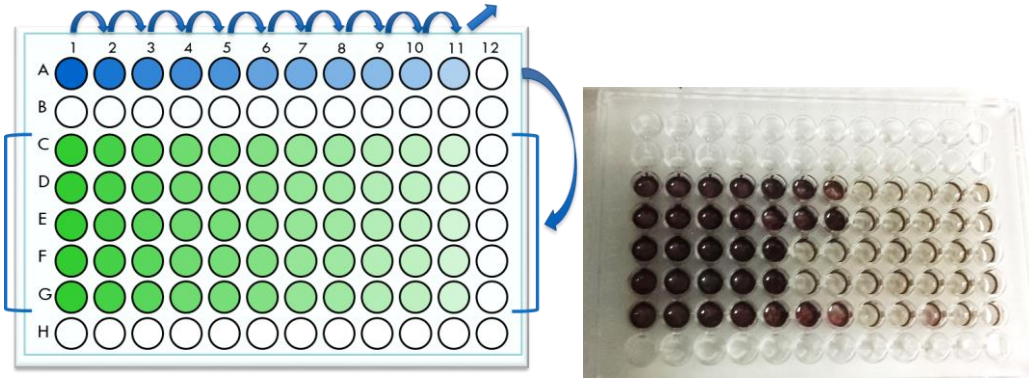

Figure 6 - (a) Schematic representation of a MPN 96-well plate, where the tenfold dilutions are applied (line A); (b) Example of a MPN plate after 15 days of incubation and coloration with INT.

2.2. Microcosm bioremediation study

A microcosms experiment was performed to evaluate the efficiency of the bacterial consortium obtained from the mixture of the 5 isolated bacterial strains (MIX, obtained as described in section 2.1) to degrade petroleum HC through the application of different remediation techniques. In this experiment, 4 treatments were tested: natural attenuation, biostimulation, and a combination of biostimulation and autochthonous bioaugmentation (BS + ABA). Two bioaugmentation treatments were tested, one with the MIX consortium previously enriched with sodium acetate as a carbon source and the other with the MIX consortium previously enriched with petroleum as a carbon source (as described in section 2.1).

2.2.1. Microbial growth rate optimization

Preceding the enrichments with petroleum and sodium acetate, the microbial growth curve, using sodium acetate, was evaluated during 7 days.

This optimization was carried out in 250 mL sterilized glass flasks with 25 mL of sterilized BH medium inoculated with the MIX consortium and a final concentration of 1 g L^{-1} of sodium acetate. For this optimization assay, 2 conditions were tested: flasks with daily input of sodium acetate solution (A) and flasks with addition of sodium acetate solution twice a week (B), achieving a final concentration of 1 g L^{-1}. Both conditions were tested in duplicates. Flasks were kept with constant agitation and at constant temperature (100 rpm; 28ºC). The solution OD was determined every day as before in a spectrometer at 600 nm.

2.2.2. Enriched 5 bacterial strains mixture consortium

Considering the results of the microbial growth curve (section 2.2.1) and the enrichment experiments with 4 different carbon sources (section 2.1.4), two 4-day enrichment processes, one with petroleum (P) and another with sodium acetate (A), were carried out before the microcosms experiment. The enrichments are important so that bioremediation tests can start with a high density of microorganisms, towards the enhancement of HC biodegradation.

A MIX consortium was prepared with loops of biomass collected from culture plates. For that, colonies of each initial bacterial strain (CPN 1 to 5) (equal amounts of each strain) were re-suspended in 3 mL of sterile BH medium (section 2.1). After homogenization of the mixture, the OD was adjusted to ca. 1, intending for a high initial microbial density.

The enrichments were performed in 250 mL sterilized glass flasks with 20 mL of sterilized BH medium for the flasks with sodium acetate and 40 mL for the flasks with petroleum, both in triplicates. In the petroleum flasks, 1 mL of sterilized petroleum was added. Regarding the enrichment with sodium acetate, sterilized sodium acetate solution was added daily, ensuring a final concentration of 1 g L^{-1}. For petroleum enrichment the volume of BH medium was doubled in order to get as much biomass as possible from each enrichment.

After 15 days, the 3 flasks of each treatment were mixed into a composed sample and its content was centrifuged. The supernatant was discarded and the pellet re-suspended in 1 mL of unsterile natural seawater, collected in Matosinhos beach, resulting in an inoculum for the following microcosms experiment (section 2.2.3).

2.2.3. Microcosms experiment

The microcosms were prepared in 100 mL sterilized glass flasks, all with 20 mL of seawater (SW) collected from a Matosinhos city beach, and 0.5 mL of sterilized petroleum (P). Four treatments were performed, natural attenuation (N); biostimulation (BS); combination of biostimulation and bioaugmentation with the MIX consortium enriched with sodium acetate (BS+ABA (EA)); and combination of biostimulation and bioaugmentation with the MIX consortium enriched with petroleum (BS+ABA (EP)) (Figure 7). Triplicate flasks were prepared for each treatment.

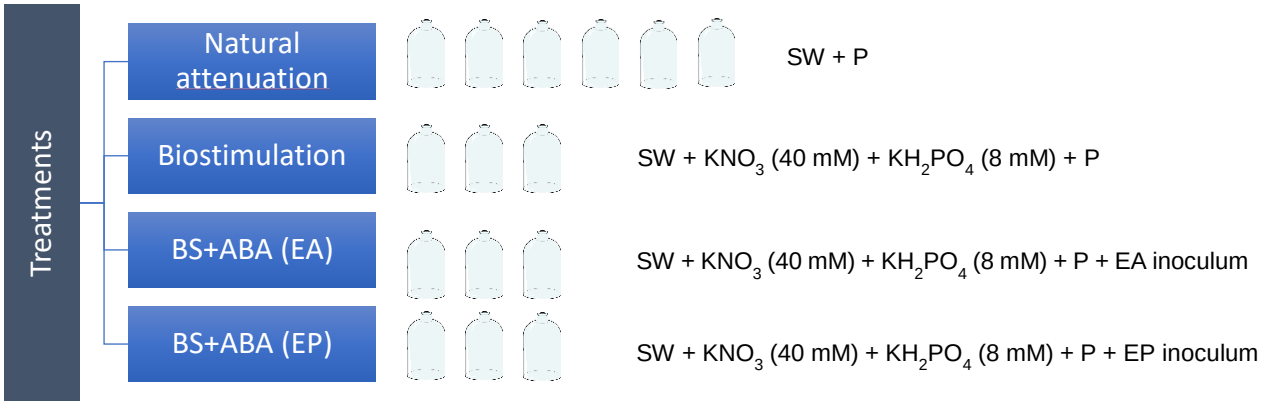

Figure 7 - Scheme of microcosm experiment with the different treatments: natural attenuation (N), biostimulation (BS), combination of biostimulation and autochthonous bioaugmentation with consortium enriched with sodium acetate (BS+ABA (EA)) or with petroleum (BS+ABA (EP).

For the N treatment, 6 flasks were prepared, being 3 of them removed at the beginning of the assay, to be used as initial microcosm samples (T0) for MPN and Total Petroleum Hydrocarbons (TPHs) determinations.

Many times, bacteria lack essential nutrients like nitrogen and phosphorous to perform bioremediation of HC. Nutrients were added in the form of potassium nitrate (KNO_3) and potassium di-hydrogen phosphate (KH_2PO_4) together with the carbon source from petroleum in a final ratio of C/N/P of (100:10:1), as described in Almeida *et al* (2013), for optimal bioremediation rates.

Before the preparation of the sterile nutrients solutions, filtered through 0.2 µm sterile filters, KNO_3 was dried at 60 ºC, for several hours, whereas KH_2PO_4 was left to dry overnight at 110 ºC, to remove water.

The flasks were incubated open, in the dark, for 15 days at room temperature, with orbital agitation (100 rpm) without changes of medium or flasks and no additional carbon source input. In addition, flasks were shaken manually every day, to ensure a better homogenization.

At the end of the 15 days, samples solutions were collected for MPN and CFU calculation and further bacterial strains isolation. The flasks and the remaining solutions were stored at -20 ºC for TPHs assessment.

2.2.4. Abundance of hydrocarbon degraders by the most probable number method

MPN was accessed in 96-well plates, as described formerly in section 2.1.5, with the exception that the final samples of the experiment (T15) were diluted in BH medium, starting the A1 well with a dilution of 10^{-6}.

2.2.5. Total petroleum hydrocarbons concentrations

A previously optimized procedure (Almeida *et al*. 2013) was used.

After unfreezing the samples, 1 mL of each replicate was mixed with 10 mL of tetrachloroethylene (>99%, from Sigma) and extracted in an ultrasonic bath (Elma, Transsonic 460/H model) for 15 min, except for N samples, where 5 mL were extracted.

After 10 min rest, the organic phase was passed to a new glass vial, containing 0.3 g of activated silica 2 % (m/m), to remove any lipids and greases that might interfere with the analysis, and 1 g of $NaSO_4$ to eliminate any water from the organic extract. Vials were agitated for 10 min in a head-to-toe shaker (Unitronic). Next, samples were filtrated through a 0.15 g glass wool tube to new glass vials.

As empty microcosm flasks presented petroleum attached to their walls, 10 mL of tetrachloroethylene were added to each flask and ultrasonic extracted as described above. The extracts were treated as described above.

The quantification of TPHs in samples extracts was carried out by Fourier transformed infrared spectroscopy (FTIR) (Jasco FT/IR-460 Plus), with a 1 cm quartz cell. Quantification was carried out by external calibration with standard solutions prepared in tetrachloroethylene.

To prepare standard solutions, two stock solutions were prepared in advance, Stock1 with 100 μL of hexadecane and 100 μL of isooctane in 5 mL of tetrachloroethylene and Stock2 with 100 μL of the Sock1 solution in a final volume of 5 mL of tetrachloroethylene. Then, from the Stock2 solution, several dilutions were applied to prepare the necessary standard solutions (Table 2).

Table 2 - Composition of the standard solutions used in total petroleum hydrocarbons (TPHs) determination.

Standard solutions	Composition (in a final volume of 5mL)
ST1	200 μL of Stock 2 + tetrachloroethylene
ST2	400 μL of Stock 2 + tetrachloroethylene
ST3	650 μL of Stock 2 + tetrachloroethylene
ST4	900 μL of Stock 2 + tetrachloroethylene
ST5	1300 μL of Stock 2 + tetrachloroethylene

This technique allows us to quantify TPHs concentrations, by comparing directly to the calibration curve and using peak heights at 2925 cm^{-1} in the infrared spectrum.

The TPHs concentrations were the sum of the TPHs from the empty flasks and the TPHs concentration determined for each sample solution.

2.2.6. Colony-forming units (CFU) and isolation of bacterial strains

At the end of the microcosms experiment, a composed sample was taken from each treatment to a 1 mL sterilized microtube and diluted in sterilized saline solution (85 %), and further dilutions were applied for the counting of colony-forming units (CFU mL^{-1}). Tenfold dilutions were applied and 100 μL of each dilution from 10^{-3} to 10^{-7} were inoculated in PCA medium plates, for the N, BS and BS + ABA (EA) treatment, while regarding BS + ABA (EP), 10^{-5} to 10^{-7} dilutions were inoculated.

After incubation at 28 ºC for about 3 days, a growth of colonies between 30 and 300 were counted and its original dilution registered. For each treatment, colonies with different morphologic characteristics (color, shape, size, transparence, etc.) were replicated by the streaking method, for further bacterial strains isolation and identification.

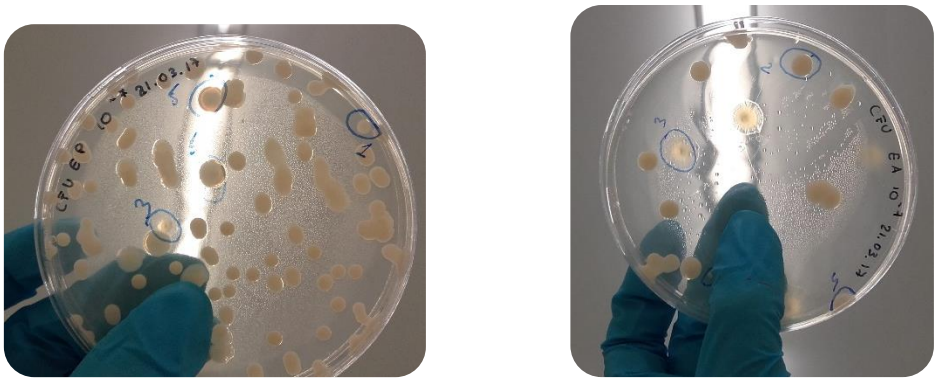

Figure 8 - Example of a CFU counting plate, from the ABA treatments, one with the EA inoculum (on the left) and another with the EP inoculum (on the right), where different morphological colonies were selected for streaking methods, and further identification.

2.2.7. Phylogenetic analysis of the bacterial strains

2.2.7.1. DNA extraction and quantification

DNA was extracted with the E.Z.N.A.® Bacterial DNA Kit (Omega, bio-tek). The quantification of the extracted DNA was evaluated by the kit Quant-it HsDNA and quantified in the Qubit fluorometer (Invitrogen).

The quantification values were accessed by a comparison to the calibration curve, made of 2 standards solutions, S1 and S2, included in the kit. These solutions were made with 190 μL of the work solution, previously prepared and 10 μL of the standard solution, with a 2 min reaction period, and after that time, values were read in the Qubit fluorometer. The work solution was made in a 15 mL tube, with 199 μL of buffer and 1 μL of Qubit™ dsDNA HS reagent. For the extracted samples, 2 μL of each sample was mixed with 198 μL of the work solution in a 0.5 mL microtube, and left to rest, again, for 2 min before reading its value on the qubit fluorometer. No solution chemical compositions are provided by the kit manufacture. All this procedure was performed in a low light environment to prevent alterations in the reagents (photodegradation).

2.2.7.2. PCR analysis and electrophoresis gel

After the defrosting of the extracted samples, the amplification of the V1- V9 regions of the bacterial 16S rRNA gene, by the Polymerase Chain Reaction (PCR), with the universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') was proceeded.

An initial 2x QIAGEN Multiplex PCR Master mix was made, containing 5 μL of Qiagen and 1.5 μL of each universal primer.

Before the sequencing of the samples, a final volume of 10 μL of PCR samples was applied in the 0.5 mL PCR tubes. For that purpose, in each sample tube, 7 μL of the QIAGEN Multiplex PCR Master mix was blended with 2 μL of the extracted DNA sample and 1 μL of DNA-free water. A control sample was also done, replacing the 2 μL of extracted DNA, by DNA-free water.

The samples were incubated in a 96-well thermal cycler, with the following program: an initial cycle of 15 min at 95 ºC, then 35 cycles at 94 ºC for 30 min, followed by 30 cycles 48 ºC for 1h 30min, 35 cycles at 72 ºC for 2 min and a final cycle of 10 min at 72 ºC.

An agarose gel was made dissolving 1.5 g of agarose in 100 mL of TAE (Tris-acetate-Ethylenediamine tetraacetic acid), and heated in the microwave for 4 min. Afterwards, 0.5 μl of SYBR® safe was added and the agarose solution was let settle in a cassette molder for 30 min.

Later the agarose gel was placed in a horizontal electrophoresis, where 3 μL of each sample, control and GRS Ladder 1 Kb was added to the wells. They were exposed to an electric flow of 150 V for 30min, and at the end the gel was observed, along with the DNA bands in the zone of 1500 bp. Next 7μL of the resulting PCR products were sent to the I3S institute for sequencing by sanger sequence.

2.3. Data and statistical analyses

Triplicates of MPN and TPHs concentrations were analyzed and their mean values (n=3) and standard deviations were determined.

For both parameters, statistical analysis was made with the STATISTICA program (version 13.2), where a parametric Student's t-test, with the mean values and their

standard deviations, was applied. Significant differences were considered, when p values were equal or below 0.05. MPN/mL values were previously transformed with base 10 logarithm.

After the sequencing of bacterial strains, sequencing results were analyzed with Geneious (version 4.8.2), and the consensus sequence extracted and run through the ncbi genbank, and two other databases, for a more precise research and comparison, ezbiocloud (http://www.ezbiocloud.net/identify) and rdp database (https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Chapter 3
Results

3. Results

3.1. Hydrocarbons-degrading bacterial consortium

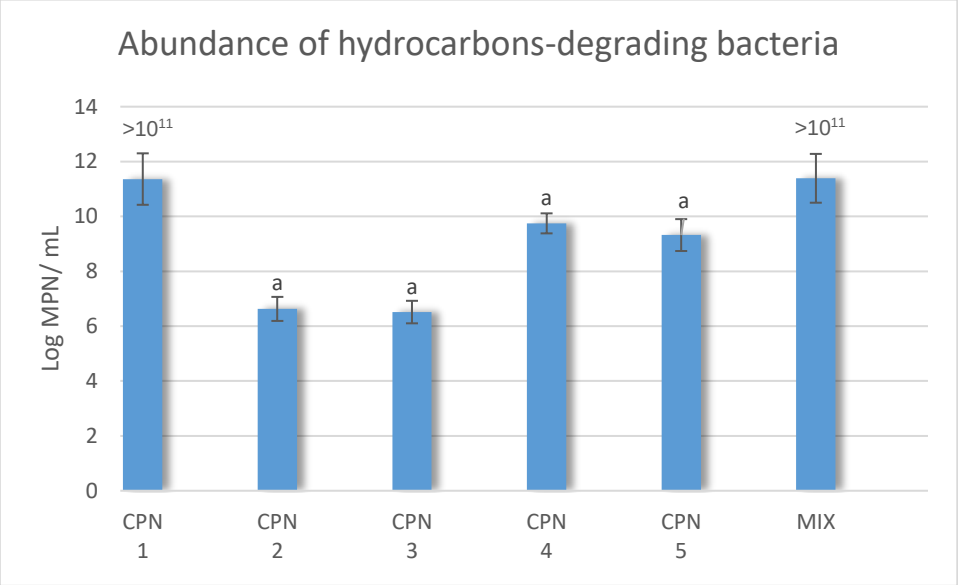

Figure 9 - Abundance of hydrocarbons--degrading bacteria for each of the isolated bacterial strains, (CPN 1, 2, 3, 4, 5) and for the mixture of the 5 bacterial strains (MIX) (mean values, standard deviations, n=3) evaluated by the most-probable number (MPN) method. a - represents significant differences comparing with the MIX ($p \leq 0.05$).

The hydrocarbons-degrading capacity of each isolate bacterial strain was tested alone and in a mixture (MIX), by MPN method. After 15 days of incubation, bacterial strains CPN 2 to 5 had densities of hydrocarbon degraders between 10^6 and $>10^9$ MPN/ mL. The bacterial strain CPN 1 and the MIX showed densities $>10^{11}$ MPN/ mL.

All the bacterial strains and the MIX had the ability to degrade petroleum HC. All the bacterial strains, apart from CPN1, showed statistically significant differences ($p \leq 0.05$) comparing to the MIX.

Although no significant differences were seen between CPN1 and the MIX, the MIX was chosen for further studies, since distinct species can degrade different petroleum HC.

3.2. Enrichment experiments with 4 different carbon sources

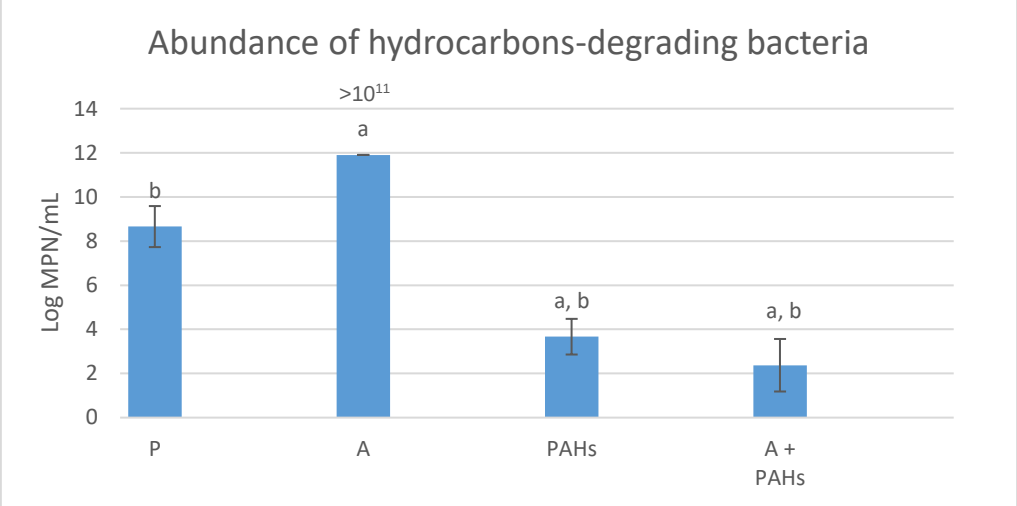

Figure 10 - Abundance of hydrocarbons degraders for the bacterial strains mixture (mean values, standard deviations, n=3) when different carbon sources were supplied: Petroleum (P), Sodium acetate (A), a mixture of polycyclic aromatic hydrocarbons (PAHs) and a combination of sodium acetate with the mixture of PAHs (A + PAHs), evaluated by the most-probable number (MPN) method. a – significant differences comparing with the P treatment; b - significant differences comparing with the A treatment.

The enrichments with P and A presented high densities of hydrocarbon degraders, respectively 10^8 and $>10^{11}$ MPN/mL.

The mixture of PAHs alone or in the presence of sodium acetate could not enhance hydrocarbon-degraders abundance. No significant differences were observed between this two last groups, with hydrocarbons degraders abundances significantly ($p<0.05$) lower than those observed in the P and A treatments.

The consortia enriched with P and with A were selected to be used in the microcosms experiment.

3.3. Microbial growth rate optimization

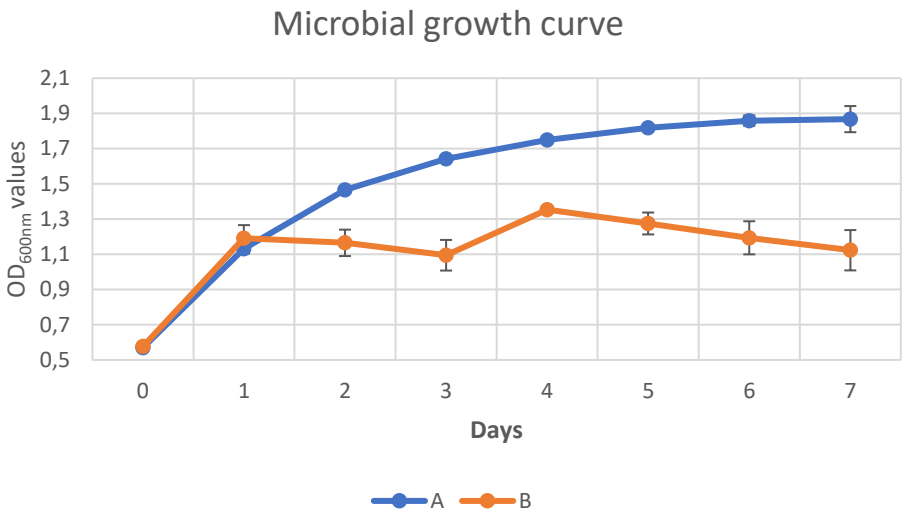

Figure 11 - Microbial growth rate of the mixture of 5 isolated bacterial strains, when sodium acetate was added to the flasks daily (A) and twice a week (B) (mean values, standard deviation, n=2).

The treatment where sodium acetate was added daily (A), presented higher density of bacteria along time, comparing to the treatment where sodium acetate was added only twice a week (B) (Figure 11).

The maximum growth was detected at the end of the experiment (day 7) with an OD close to 1.9, where the curve growth rate started to decrease. A four-day enrichment was selected to be used for the microcosms experiment, with addition of sodium acetate daily, since at this time period the microbial growth was in the exponential phase.

3.4. Microcosm experiments

3.4.1. Visual aspect

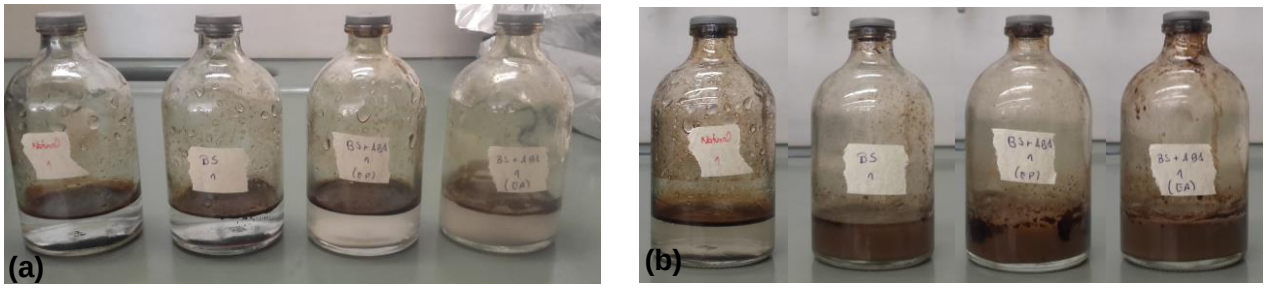

Figure 12 - Visual aspect of the microcosm flasks at the beginning (a) and after 15 days (b) of the experiment, in which different treatments were applied: Natural attenuation (N), Biostimulation (BS), a combination of Biostimulation and Bioaugmentation with a consortium enriched with sodium acetate (BS + ABA (EA)) and a combination of Biostimulation and Bioaugmentation with a consortium enriched with petroleum (BS + ABA (EP)).

Photos of the microcosm flasks (Figure 12) were taken at the beginning and at the end of the experiment, after flasks were removed from the agitator, and after their stabilization.

At the end of the microcosms experiments (Figure 12b), it was possible to observe that the petroleum was blended with medium in the flasks of the BS, EA and EP treatments. For N treatment, a clear separation between the oil slick and the remaining medium, clearly observed at the beginning of the experiment, was stills very clear at the end of the experiment. At the end of the experiment, the flasks of the treatments BS and BS + ABA were similar, with a homogeneous solution as petroleum blended with the medium. Although they were similar at the end of the experiment, the blending in flasks of BS + ABA was observed earlier, after one week after incubation

3.4.2. Abundance of hydrocarbons-degrading bacteria

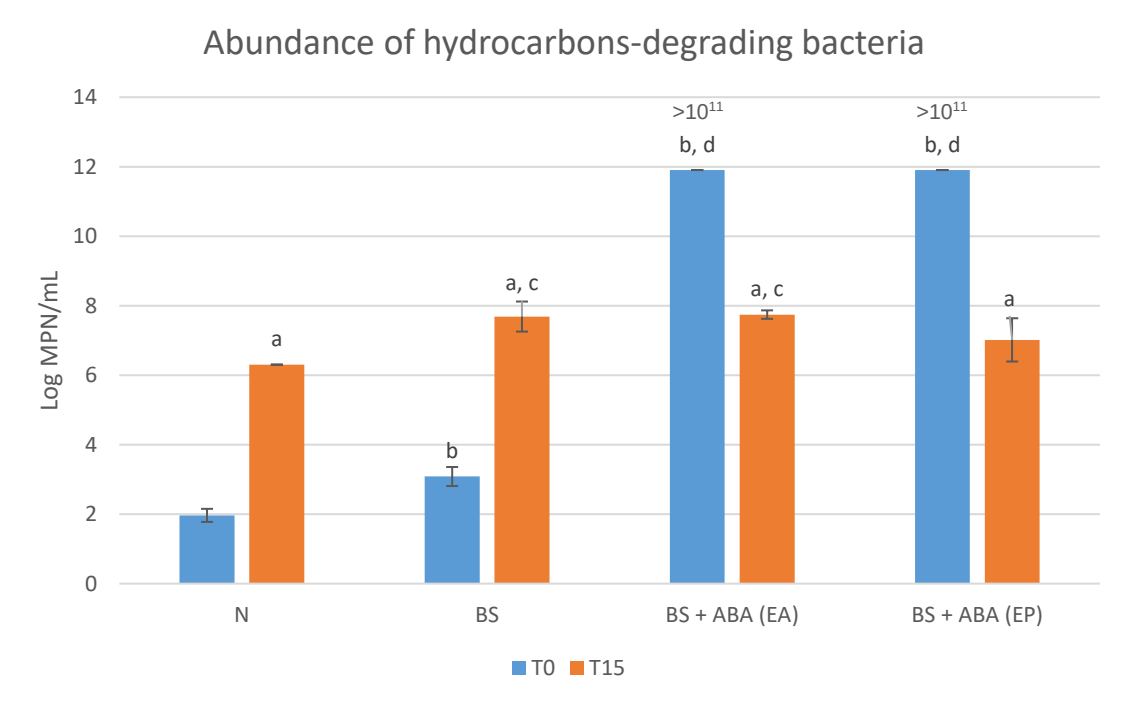

Figure 13 - Abundance hydrocarbons degraders at the beginning (T0) and after 15 days (T15) of the microcosm experiment (mean values, standard deviations, n=3) in which different treatments were applied: Natural attenuation (N), Biostimulation (BS), a combination of Biostimulation and Bioaugmentation with a consortium enriched with sodium acetate (BS + ABA (EA) and a combination of Biostimulation and Bioaugmentation with a consortium enriched with petroleum (BS + ABA (EP)), evaluated by the most-probable number (MPN) method. a - significant differences between T0 and T15; b- significant differences comparing all treatments with N in T0; c- significant differences comparing all treatments with N in T15; d- significant differences comparing BS with the ABA treatments in T0.

At T0, the treatments BS + ABA (EA) and BS + ABA (EP) presented higher abundance of hydrocarbons degraders (MPN/mL > 10^{11}). Initial values of hydrocarbons degraders in the natural attenuation (N) and the biostimulation (BS) treatments revealed the presence of hydrocarbons degraders in the natural seawater used in the experiment (Figure 13).

The treatments N and BS increased their hydrocarbons-degraders abundance by the end of the microcosm assay. All treatments showed significant differences, within the groups, comparing the beginning with the end MPN values.

At the end of the experiment, no significant differences, in terms of hydrocarbons degraders abundance were observed between the treatments BS, BS + ABA (EA) and BS + ABA (EP).

3.4.3. Hydrocarbons removal

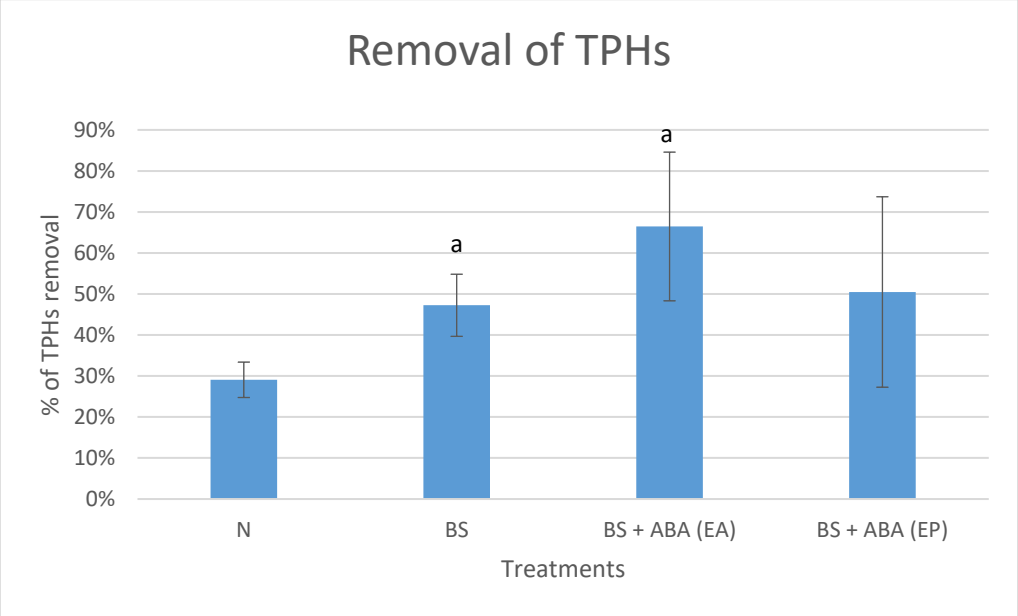

Figure 14 - Removal percentage of total petroleum hydrocarbons (TPHs), (mean values, standard deviations, n=3) within the microcosm treatments: Natural attenuation (N), Biostimulation (BS), a combination of Biostimulation and Bioaugmentation with a consortium enriched with sodium acetate (BS + ABA (EA) and a combination of Biostimulation and Bioaugmentation with a consortium enriched with petroleum (BS + ABA (EP)), accessed by the Fourier Infrared spectroscopy method (FT/IR). a – significant differences comparing all treatments with N.

At the end of the experiments, the percentage of TPHs removal was evaluated for each treatment. The treatments BS, BS + ABA (EA) and BS + ABA (EP) showed higher TPHs removal percentages than natural attenuation (N).

Although between these 3 last treatments, no significant differences were seen, the treatment in which the consortium was enriched with sodium acetate, showed the higher removal percentage (66 %) (Figure 14).

3.4.4. Bacterial strains identification and CFUs

The isolated bacterial strains CPN (1 to 5) used to develop the MIX consortium were identified and are represented in Table 3. The 5 isolates belong to 3 different genera, *Pseudomonas Rhodococcus* and *Acinetobacter* and 2 phyla, Proteobacteria and Actinobacteria.

Table 3 - Taxonomic identification of the isolates (CPN1, 2, 3, 4 and 5) used for the enriched consortia which were applied in the microcosms.

Isolates	Identification	Base pairs	Phylum
CPN1	*Pseudomonas sp.*	1401	Proteobacteria
CPN2	*Rhodococcus erythropolis*	1362	Actinobacteria
CPN3	*Rhodococcus erythropolis (strain cpn3)*	1369	Actinobacteria
CPN4	*Pseudomonas sp.*	1390	Proteobacteria
CPN5	*Acinetobacter johnsonii*	1399	Proteobacteria

Table 4 - Values of Colony-forming units (CFUs) of each microcosms treatment at the end of the experiment: Natural attenuation (N), Biostimulation (BS), a combination of Biostimulation and Bioaugmentation with a consortium enriched with sodium acetate (BS + ABA (EA) and a combination of Biostimulation and Bioaugmentation with a consortium enriched with petroleum (BS + ABA (EP)).

Dilutions	N	BS	BS + ABA (EA)	BS + ABA (EP)
10^{-3}	<30	>300	>300	--
10^{-4}	<30	>300	>300	--
10^{-5}	<30	**45**	**72**	**101**
10^{-6}	<30	<30	<30	<30
10^{-7}	--	--	--	<30
CFU/ML	--	$4.5x10^{7}$	$7.2x10^{7}$	$1.01x10^{8}$
isolates	8	10	5	5

The treatment of BS + ABA (EP) was the one that presented the highest bacterial density. The agar plates where the samples of the natural attenuation were inoculated, presented insufficient growth to calculate its CFU/mL, but it was possible to isolate some colonies, and alike other treatment agar plates, the colonies distinguished by their morphological characteristics were isolated and identified (Table 5).

Table 5 - Taxonomic identification of bacterial strains isolated at the end of the microcosms experiment for the different treatments: Natural attenuation (N), Biostimulation (BS), a combination of Biostimulation and Bioaugmentation with a consortium enriched with sodium acetate (EA) and a combination of Biostimulation and Bioaugmentation with a consortium enriched with petroleum (EP). Species identification were determined with a % of similarity higher than 99%

Isolate strain	Identification	Base pairs	Phylum
N1	Rhodobacteraceae*	1345	Proteobacteria
N2	*Pseudomonas sp.*	1399	Proteobacteria
N3	*Pseudomonas sabulinigri*	1399	Proteobacteria
N4	*Pseudomonas sp.*	1393	Proteobacteria
N5	*Microbacterium oxydans*	1382	Actinobacteria
N6	*Pseudomonas sp.*	1395	Proteobacteria
N7	*Pseudomonas aestusnigri*	1391	Proteobacteria
N8	-		
N9	*Pseudomonas stutzeri*	1387	Proteobacteria
N10	*Pseudomonas alcaligenes***	1385	Proteobacteria
BS1	*Pseudomonas taeanensis*	1337	Proteobacteria
BS2	Rhodobacteraceae*	1331	Proteobacteria
BS4	*Pseudomonas taeanensis*	1397	Proteobacteria
BS5	Rhodobacteraceae*	1350	Proteobacteria
BS7	*Pseudomonas pachastrellae*	1408	Proteobacteria
BS8	*Pseudomonas sp.*	1394	Proteobacteria
BS9	*Pseudomonas taeanensis*	1407	Proteobacteria
BS11	*Rhodococcus erythropolis*	1392	Actinobacteria
BS13	*Pseudomonas taeanensis*	1389	Proteobacteria
BS14	*Pseudomonas stutzeri*	1403	Proteobacteria
EA1	*Salinibacterium amurskyense*	1364	Actinobacteria
EA2	*Rhodococcus erythropolis*	1366	Actinobacteria
EA3	*Pseudomonas sp.*	1396	Proteobacteria
EA4	*Acinetobacter johnsonii*	1408	Proteobacteria
EA5	*Pusillimonas sp.*		Proteobacteria
EP1	Rhodobacteraceae*	1332	Proteobacteria
EP2	*Rhodococcus erythropolis*	1363	Actinobacteria
EP3	*Pseudomonas sp.*	1384	Proteobacteria
EP4	*Pusillimonas sp.*	1405	Proteobacteria
EP5	*Acinetobacter johnsonii*	1400	Proteobacteria

** the identification of the isolates was made only until the family level;*
*** the isolate may represent a new species, with 98% of similarity.*

All the identified species, resulting from the microcosms experiments, survived the petroleum-contaminated environment and can use petroleum HC as energy source.

It was observed a predominance of the Proteobacteria phylum, among every treatment (Table 5). Within these phylum, species form the genus *Pseudomonas* are the major group represented.

Also, it was possible to observe that all the bacterial strains that were used to prepare the ABA consortia (Table 3) could be recovered in the respective treatments at the end of the experiment (Table 5).

For the isolate N8, it was not possible to obtain an identification, due to the poor quality of the sequenced PCR product.

For the isolates N1, BS2, BS5 and EP1, it was not possible to reach a species nor genus identification, although 3 data bases were used. The search revealed that these isolated bacterial strains, belonging to the Rhodobacteraceae family, present correspondence to 3 genera (*Albirhodobacter marinus*; *Pseudohodobacter sp*. and *Rhodobacter sp.*). This might have happen given the short length of the sequences or the DNA regions sequenced.

Chapter 4

Discussion and conclusions

4. Discussion and conclusions

4.1. Discussion

Bioremediation techniques for the recovery of oil spill incidents are nowadays a focus of investigation. An average clean-up of oil contaminated habitat with the use of chemical dispersants or mechanical removal can be very expensive due to the amount of resources and personnel required. Though these treatments are important to rapidly control the diffusion and drift of the oil, they are not suitable for ecological restoration and, in some cases, they can even present threats to marine ecosystem. So, bioremediation has been recurrently investigated as an efficient, eco-friendly and cheap alternative for remediation of oil-polluted sites (Al-Majed *et al.*, 2012; ITOPF).

There is a known ability of natural microbial communities to degrade petroleum HC, that can be enhance by using bioremediation technologies. For instance, Medina-Bellver *et al.* (2005) found evidence of crude oil degradation in samples collected from the Galician shore, an area impacted by the Prestige oil spill, through the analyses of dissolved inorganic carbon isotopic shifts. Further biostimulation tests with seawater from that location with added crude oil and different ratios of N/P, throughout 1 month at 20 ºC and 150 rpm, proved the biodegradation ability of the indigenous bacterial community.

Reis *et al.* (2014) also tested the ability of an indigenous microbial community, of an impacted beach from the Prestige oil spill, to degrade petroleum HC upon new contamination with crude oil in microcosms experiments. After the 15 days of the microcosms experiments, supplemented with three different nitrogen concentrations, the indigenous community proved to be efficient in the degradation of HC.

Another example of the intrinsic microbial community ability to degrade petroleum HC is demonstrated in the work of Almeida *et al.* (2013), where the indigenous microbial community, originated from sediment of a beach unimpacted by oil spills, displayed ability to degrade petroleum HC, upon contamination with crude oil, in small-scale tests.

It is important the isolation and identification of autochthonous oil-degraders from several sites, and the development of efficient hydrocarbons-degraders consortia for the degradation of petroleum HC, for future application in oil spill remediation technologies. In this way, the response to an oil spill would be more efficient and ecologic.

In the present work, 5 bacterial strains previously isolated from a microbial consortium capable of degrading petroleum HC, were used, including two strains of *Pseudomonas*

sp., two strains of *Rhodococcus erythropolis* and a strain of the species *Acinetobacter johnsonii*.

All the bacterial strains used for the development of the autochthonous bioaugmentation consortia within the present work, have shown ability to degrade HC in several other studies. *Pseudomonas* are one of the major groups indicated, thanks to their production of surfactants and superior performance in the HC degradation (Banat *et al.*, 2010; Ron & Rosenberg, 2002). *Rhodoccocus erythropolis* is a well-known oil degrading bacteria, described for their biosurfactant production and ability to degrade petroleum HC (Peng *et al.*, 2007). There was another strain in the consortia, *Acinetobacter johnsonii*, belonging to a genus group known for its ability to degrade a large spectrum of HC (Lee *et al.*, 2012).

Since these bacterial strains were isolated from samples collected near an oil refinery, they could have already a predisposition to degrade petroleum HC. In a recent study of Rocha *et al.* (2017), PAHs in concentrations harmful for the environment were detected in water and sediment samples from various locations, including 2 north-western Atlantic beaches close to the oil refinery and the port of Leixões. This contamination was associated with anthropogenic activities linked to the refinery and the port.

The analysis of the hydrocarbons-degrading bacterial abundance by MPN, for each isolated bacterial strain and their mixture, showed that all had potential to degrade petroleum HC. The mixture of the 5 bacterial strains was chosen to continue for the development of consortia.

Since crude oil is a complex mixture of HC and other compounds, and different bacterial species degrade distinct HCs, a consortium of hydrocarbon-degrading bacteria would perform better in the degradation of petroleum HC than the bacterial strains alone (Dell'Anno *et al.*, 2012; Hassanshahian & Cappello, 2013). For this reason, only bacterial consortia were tested in microcosms experiments, for their performance in the degradation of crude oil under different treatments.

Another key factor for bioremediation success is the abundance of oil-degraders in the environment after an oil spill. Since the contaminated area is extensive, a high biomass of bacteria is required and, therefore, different techniques, such as different carbon sources, for the increase of the bacterial biomass must be tested, before application in the field.

So, enrichments of the bacterial consortium (mixture of the 5 bacterial strains) with petroleum, sodium acetate, a mixture of PAHs and a combination of sodium acetate with

PAHs were carried out, for evaluating the best method to obtain high biomass of hydrocarbon-degrading bacteria.

Several studies use crude oil or other HC substrate for the enrichment phase of the consortia (Bacosa *et al.*, 2012; Santisi *et al.*, 2015; Vila *et al.*, 2010). Sodium acetate was included in the present tests, because it is a simpler form of carbon source, which is rapidly up taken by bacteria, directly into the tricarboxylic acid cycle. This compound was used before as a growth supporting substrate (Alexandrino *et al.*, 2017).

In the enrichment with the 4 different carbon sources, the mixture of PAHs either alone or in combination with sodium acetate could not enhance the hydrocarbon-degrading bacterial abundance. This might be due to PAHs high concentrations and dilution in dichloromethane, which could have been toxic to the bacterial strains, inhibiting their biodegradation rate, or because PAHs degraders could not adapt and grow as fast. In fact, aromatic HC are more difficult to degrade. The enrichments with sodium acetate and with petroleum were the ones with higher biomass recovered and higher abundance of hydrocarbons-degraders.

It was also observed that an enrichment phase with sodium acetate only, with a daily input of the substrate, provided a higher biomass of bacteria, without loss of their ability to degrade HC, reflected on the high abundance of hydrocarbon-degraders.

Considering the efficiency of the sodium acetate and the petroleum enrichments, they were selected as enrichment processes for the development of hydrocarbon-degrading consortia, which were then tested in a microcosm assay with seawater collected in a beach near by the one from where the bacterial strains were isolated. Various treatments (including biostimulation and bioaugmentation) were tested to evaluate their ability to degrade petroleum HC.

Within this study it was clear that the best performance in the microcosms experiment was observed in the treatment of biostimulation combined with bioaugmentation with the consortium enriched with sodium acetate. In fact, at the end of the 15 days experiment, hydrocarbon-degrading abundance reached 10^8 MPN/mL and the removal of TPHs was ca. 66 %. Considering that these assays occurred throughout 15 days and in the presence of the natural community in the tested seawater, the results of 66 % TPHs removal are promising.

The degradation of the petroleum HCs can occur due to hydrocarbon-degrading microorganisms present in the water column or in the sediments, once the oil reaches the sea bottom or sea shore. Many scientists used oil-degrading bacteria originated from seawater samples (Bao *et al.*, 2012; Jurelevicius *et al.*, 2013; Nikolopoulou*, et al.*, 2013)

and from sediment samples (Almeida *et al.*, 2013; Patowary *et al.*, 2016; Pontes *et al.*, 2013; Yakimov *et al.*, 2005) and apply them in bioaugmentation studies. However, the application of autochthonous bioaugmentation is generally more advantageous than exogenous bioaugmentation, since the exogenous bacterial strains might not compete with the natural microbial community, and so display less efficiency on the degradation of petroleum HC. Works like, Almeida *et al.* (2013) evidence such advantages and success of the use of autochthonous bioaugmentation.

Almeida *et al.* (2013), tested the effects of autochthonous bioaugmentation and biostimulation treatments in microcosms assays with sediment doped with crude oil (1 % (v/v)), and inoculated with a consortium originated from the same sediment. Visual inspection showed that the petroleum was more blended with the medium in the flasks of the biostimulation, bioaugmentation and their combination, comparing to the natural attenuation. These former results are in accordance with the results from the present microcosms experiment, where a homogenization of the flask content was seen for the biostimulation and bioaugmentation treatments, whereas in the natural attenuation a clear distinction of the oil layer and water column was visible.

Some other authors studied the bioaugmentation with consortia enriched with crude oil (Bao *et al.*, 2012; Malik & Ahmed, 2012; Nikolopoulou *et al.*, 2013b; Santisi *et al.*, 2015). Comparable to this work, (Nikolopoulou *et al.*, 2013b) studied the combined effect of biostimulation and autochthonous bioaugmentation treatment as well. Their tests were performed with seawater and 0.5 % crude oil, but for a period of 30 days with an enriched microbial consortium. At the end of those 30 days they observed a biodegradation rate of 77 % and 10^4 MPN/mL abundance. This biodegradation rate was slightly higher than the one observed in the present study but occur during a longer period of time. Nikolopoulou *et al*. (2013b) performed at the same time with the previous treatment, one with the addition of a biosurfactants rhamnolipid, which achieved a biodegradation rate of 90 % at the end of 30 days and an MPN value of 10^6 MPN/mL. After 15 days of the assay they observed an abundance of strains of some genus like *Pseudomonas, Marinobacter*, and *Alcanivorax*, while at the end of the 30 days, *Pseudomonas* was one of the dominant ones. These results are in accordance with the present study, where *Pseudomonas* genus was one of the most represented group at the end of the microcosms experiment.

Bao *et al*. (2012) also performed microcosm assays to determine the oil-degrading capacity of 4 bacterial strains previously isolated from contaminated water and soil, to degrade crude oil, amongst other HC substrates. For the crude oil trials, the mixed consortium was inoculated in mineral salt medium in the presence of crude oil (0.5 w/v)

for 14 days, with samples collected for OD calculation. The consortium was composed by 4 bacterial strains, in which 3 belonged to the same species (*Brevibacillus parabrevis*), making it a simple structural microbial community. They observed that, with an increase of the incubation time, there was an increase of the degradation rate, and at the end a degradation rate of 79 % was achieved, as measured by UV spectrophotometry.

Alternatively, in the present study, the analysis of the TPHs was made by the FTIR method, described in the protocol Couto *et al.* (2013) and considered to be an effective, quick and cheap method.

Jurelevicius *et al.* (2013) tested the effect of nutrient addition (C/N/K/P; 100:10:1:1) in microcosms, to aquatic samples contaminated with different carbon sources, including microcosms assays with 25 mL of unsterilized seawater and 1 % of crude oil addition. After a 32 day-period incubation, the biostimulation treatment showed 50 % degradation of TPHs, by GC-MS method, in comparison to the control conditions (seawater plus crude oil). Further isolation of bacterial strains in different agar medium and their identification by partial 16S rRNA gene sequences was done. At the end of the microcosms experiments, there was a predominance of the order Oceanospirillales, the *Marinobacter* and *Mesoflavibacter* genera and also an increase of the *Pseudomonas* genus, a genus also present in the current study. These results are comparable to the ones that were achieved in the present work in the biostimulation treatment, where we also achieved almost 50 % of TPHs removal at the end of 15 days incubation.

Reis *et al.* (2014) also performed biostimulation microcosms experiments in 50 ml flasks with 10 mL of sediment, containing the indigenous microbial community, 20 mL of BH medium and 0.5 mL of crude oil, for 15 days, at room temperature and constant agitation. The natural attenuation, was compared with two biostimulation treatments, one with the addition of 20mM of nitrogen and the other with 40mM of nitrogen. At the end of the experiments, it was observed an enhancement of the degradation of TPHs and PAHs in the biostimulation treatments. Regarding PAHs, degradation rates of 46 % and 70 % were observed, respectively, for the 20mM N and for the 40mM N flasks, while natural attenuation displayed only 38 % degradation.

In the present work, the bioaugmentation with sodium acetate enrichment proved to be the most effective treatment in the degradation of HC achieving, as mentioned, ca. 66 % removal of TPHs, a percentage higher than the one observed for the biostimulation treatment (ca. 47 %). Both treatments were more efficient than natural attenuation that displayed only ca. 29 % degradation.

In the present work, at the end of the microcosms experiment, the abundance of oil-degraders was estimated by the most probable number method. The 3 treatments of biostimulation and bioaugmentations, showed an abundance around 10^7 MPN/mL, with no significant differences. The decrease in the abundance of hydrocarbon-degraders in the bioaugmentation treatments, from the beginning of the experiment (higher than 10^{11} MPN/ mL) to the end, might be related to the decrease in the hydrocarbons amounts (their carbon source).

Li *et al.* (2016) performed isolation of native bacterial strains and tested their ability to degrade crude oil HC, on the way to develop 3 different consortia based on the performance of each isolate. The 3 developed consortia were tested in microcosms sets, incubated at 30 ºC with 200 rpm agitation for 7 days, being the degradation of HC estimated by gas-chromatography. The application of the consortia and the isolates alone in the microcosms displayed different effectiveness in the degradation of crude oil. The most effective consortium was the one composed by strains from the genus *Bacillus* and *Alcaligenes* with ca. 52%. of crude oil degradation. It was observed that all the isolated strains could degrade n-alkanes, but not all of them could degrade PAHs.

Although they tested different consortia, their best performing consortium was composed by only two bacterial strains, which might not be as effective in a real life oil-spill scenario due to the low diversity. Comparatively, in the present work, an enriched consortium, composed of 5 bacterial strains, was able to degrade about 66 % of total HC.

Some scientists are focusing on the production and application of biosurfactants, with high rates of HC degradation, but its possible toxicity to marine organisms is not well-known (Silva *et al.*, 2014). For instance, Nikolopoulou and Kalogerakis (2008) studied the effect of the biosurfactants and natural lipophilic nutrients (uric acid, lecithin) in the degradation process of crude oil. They performed microcosms assays with seawater, crude oil, nutrients and a biosurfactants (rhamnolipids), for 18 days at constant agitation. After 18 days, they achieved a maximum biodegradation rate of 96 %. Although its high effectiveness on the degradation of HC, the production cost of biosurfactants is very high (Banat *et al.*, 2010; Makkar *et al.*, 2011), making the use of this option alone in the bioremediation process an expensive technology.

So, there is a need for the continuous research of bioremediation technology, since it represents an effective, ecological and economical alternative. Therefore, research is needed before applying to oil spills real-scenarios, especially in ways to produce bacterial biomass in large-scale and ways to recover and identify the consortia strains at the end of a bioremediation process.

At the end of the microcosms experiment, samples from the bioaugmentation treatments were cultured in agar plates and bacterial strains identified, to observe if the strains of the consortia used in these treatments were present at the end of the assay.

The bacterial strains from the initial consortium used in the bioaugmentation treatment were present in the end of the assay, indicating the survival and growth of these strains and their possible contribution to the observed HC degradation. This was particularly notorious in the treatment that used the bacterial consortium enriched with acetate (BS+ABA+EA), where all the bacterial strains present in the initial consortium were recovered at the end of the microcosms experiment with plating in agar mediums. This was also the treatment that displayed the highest HC removal rates. Some studies also observed the maintenance of bacterial strains (Jurelevicius *et al.*, 2013; Vila *et al.*, 2010) at the end of the biodegradation experiments.

But at the end of the experiment more bacterial strains than the ones present in the added consortia were present. Once the microcosm experiment used non-sterilized natural seawater, some bacterial strains originated from the seawater natural community were expected to be recovered at the end of the experiment. An example is the growth of the strain *Rhodococcus erythropolis* in the platings from the biostimulation treatment, that could only be originated from the natural seawater microbial community.

So, independently of the initial bacterial consortium and bacterial strains added to the remediation process, changes in the microbial community structure always occur. Thus, it is important the selection of oil-degrading bacteria originated from the site to be remediated and the development of an efficient autochthonous consortia under similar conditions to the ones existent in the impacted environment.

Small-scale tests, like microcosms are the commonly practice in biodegradation assays, but there are also some mesocosms and field tests studies.

For instance, medium-scale mesocosms were used by Hassanshahian *et al.* (2014) to test the effect of biostimulation and bioaugmentation treatments. The experiments were carried out in rectangular tanks filled with 10000 L of natural seawater, with a pump to assure the oxygenation. The tanks were contaminated with 1L crude oil, and the addition of nutrients and two different bacterial strains, belonging to the species *Alcanivorax borkumensis* and *Thalassolituus oleivorans*, was tested. At the end of 20 days, the 3 different treatments (M1- biostimulation, M2- single strain bioaugmentation, M3 – consortium augmentation), achieved degradation of alkanes of 80, 95 and 70%, respectively.

Moreover, the effect of bioremediation in a simulated oil-spill incident have been demonstrated by some works (Garcia-Blanco *et al.*, 2006; Swannell *et al.*, 1999; Tsutsumi *et al.*, 2000), with evidence of the success of the bioremediation treatments implemented.

Field tests and *in situ* application of bioremediation techniques need to be considered and be target of a continuous research, because small-scale experiments may not predict with accuracy the behavior and fate of an introduced microbial consortia or other bioremediation techniques in bioremediation actions in an oil-contaminated environment (Tyagi *et al.*, 2011). One of the challenges of bioremediation technologies is the up-scaling, when one proceeds from small-scale microcosms to a higher and more realistic one, or even to field applications. The difficulty lies on the environmental conditions, water volume, resources and the larger degradation period, similar to a real-life scenario.

For instance, Bao *et al.* (2012) performed microcosms assays, described before in this chapter where ca. 79 % HC removal was achieved, and then passed to mesocoms tests in 1.5 m (L) x 0.8 m (W) x 0.7 m (H) size containers with an underwater aerator, to promote the oxygenation. Each tank was filled with 600 L of artificial seawater, contaminated with crude oil 0.5 L/m^2 and inoculated with 2.4 L of fermented bacteria. The experiment lasted 144 days. At the end of the assay it was observed a HC degradation rate of ca. 51 %, a lower value compared to the microcosm assay, probably due to the escalation process from microcosms to mesocosm test.

So, despite the promising results obtained for the bacterial consortia used in the present study for petroleum HC biodegradation, tests are still necessary to optimize their ability in more realistic scenarios.

4.2. Conclusions and Future Perspectives

There is an intrinsic capacity of the microbial community in the ocean, to degrade petroleum HC, which can be enhanced by bioremediation processes, like biostimulation and bioaugmentation.

With this work, we developed a consortium of autochthonous hydrocarbon-degrading bacterial strains and optimize their enrichment process with different carbon sources. In microcosm experiments, we demonstrated that the combination of biostimulation and bioaugmentation with a consortium enriched with acetate can be an efficient bioremediation treatment for the degradation of petroleum HC.

Heading towards the development of an efficient hydrocarbon-degrading consortium a subsequent scale-up process of the mixture of the isolated bacterial strains, enriched with sodium acetate might be considered, before applying to the field, in future remediation processes.

A subsequent library of native oil-degrading bacteria could also be done throughout different regions, and the final product, native oil-degrading consortia, might be implemented by the respective authorities responsible for the remediation of oil-polluted habitats when a particular environment is impacted by an oil spill.

In this way, with the application of an autochthonous bioremediation technique, we can face an oil spill disaster with an eco-friendly efficient and also economic approach, which not only removes the petroleum, but also remediates the polluted environment and restore its functions.

Chapter 5
References

5. References

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